

Human Rhinovirus at Naturally Occurring COPD Exacerbation

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A thesis submitted for the degree of Doctor of Philosophy at
UCL, 2014.

I, Síobhán Nicole George, confirm that the work presented in this thesis is
my own. Where information has been derived from other sources, I
confirm that this has been indicated in the thesis.

A handwritten signature in black ink, appearing to read 'SN George'.

Abstract

Chronic obstructive pulmonary disease (COPD) is an inflammatory condition of the lung caused by an abnormal response to particles and noxious gases, primarily cigarette smoke. Patients suffer daily symptoms and can have episodes of worsening symptoms termed acute exacerbations. Exacerbations are associated with impaired quality of life, faster lung function decline, higher mortality and increased risk of hospitalisation. The aetiology of COPD exacerbations is controversial; however respiratory viral and bacterial infections are an important feature of exacerbations.

This study utilised real-time qPCR to measure prevalence and load of human rhinovirus (HRV) in stable COPD and during the time-course of naturally occurring exacerbations and their recovery. HRV was assessed in association with upper respiratory tract (URT) symptoms namely cold symptoms and sore throats, secondary bacterial infection, patient reported outcomes and exacerbation frequency. Additionally, respiratory syncytial virus (RSV) was semi-quantitatively examined in stable COPD and at exacerbation.

The original contribution of this work to the field is that HRV prevalence and load are highest at exacerbation presentation and decrease during recovery. HRV load is higher in the presence of URT symptoms compared to the load without, and the load remains higher for longer with both symptoms compared to only one. This study described novel evidence for the development of secondary bacterial infection after HRV infection in natural exacerbations, and demonstrated that HRV infection is associated with patient reported outcomes. Patients with HRV had higher exacerbation frequencies compared to those without HRV. RSV prevalence did not change significantly between stable COPD and exacerbation.

The findings from this thesis have important implications in terms of exacerbation therapy. The evidence provided may allow appropriate targeting of therapeutic interventions therefore reducing exacerbation severity and frequency. These findings emphasise the importance of rapid development of therapeutic targets for the prevention and treatment of HRV infection in COPD patients.

Acknowledgements

First and foremost, I would particularly like to thank my primary supervisor, Professor Wisia Wedzicha for her unfailing support, enthusiasm and encouragement throughout the last few years; both her wisdom and inspiration have been invaluable. I also wish to thank my secondary supervisor Dr Gavin Donaldson, for his advice and help during my time in the department. Without Beverly Kowlessar and the clinicians, Dr Anant Patel, Dr Alex Mackay, Dr Richa Singh, Dr Simon Brill and Dr James Allinson, the London COPD cohort would not function. The time and care they devote to the welfare of the COPD patients makes it possible for studies such as this one to be carried out. Similarly, without Ray Sapsford and his assistance with sputum sample processing, this thesis would not have been possible. I thank the patients in the London COPD cohort for contributing so much of their time and energy in helping to try and tackle COPD. I would like to say a special thank you to Dr Davinder Garcha and Dr Anant Patel for all the advice and encouragement they have given me throughout this study. The time they have dedicated to help me has been invaluable, something for which I will be forever grateful. Thanks go to Dr Nathan Bartlett from Imperial College London, for his helpful assistance in the HRV qPCR set-up and for giving me the opportunity to shadow him and other members of his team. Of course, without my office colleagues and good friends Davinder Garcha and Ayedh Alahmari, I think some of the tough times would have not been bearable so thank you for keeping me going! Finally, I would like to dedicate this thesis to my parents Rosalyn and Brian, and also to my partner Alex, all who have put up with me during these challenging few years. Without your love and support I would not have been able to do this. I am truly thankful for everything.

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Abbreviations

AAT = α 1-antitrypsin

AE-COPD = Acute exacerbations of chronic obstructive pulmonary disease

BAL = Broncho-alveolar lavage

BHQ-1 = Black hole quencher – 1

BBQ = Blackberry quencher

CAT = COPD assessment test

cDNA = complementary deoxyribonucleic acid

CFU = Colony forming units

COPD = Chronic obstructive pulmonary disease

CRP = C-reactive protein

Ct = Cycle threshold

CXCL-10 = C-X-C motif chemokine 10

CXCL-12 = C-X-C motif chemokine 12

Cy5 = Cyanine5

DNA = Deoxyribonucleic acid

dNTP = Deoxynucleotide triphosphate

ELISA = Enzymelinked immunosorbent assay

EXACT = The exacerbations of chronic pulmonary disease tool

EXP = Exacerbation presentation

FAM = Fluorescein amidite

FEV₁ = Forced expiratory volume in one second

FVC = Forced vital capacity

GOLD = Global Initiative for Chronic Obstructive Lung Disease

GM = Growth Medium

GRO α = growth-regulated protein α

H influenzae = *Haemophilus influenzae*

HRV = Human rhinovirus

IAC = Internal amplification control

ICAM – 1 = intercellular adhesion molecule – 1

ICS = Inhaled corticosteroid

IQR = Interquartile range

IFN = Interferon

IFN- α = Interferon - α

IFN- β = Interferon - β

IFN- γ = Interferon - γ

IL-1 β = Interleukin-1 β

IL-11 = Interleukin-11

IL-1 α = Interleukin-1 α

IL-6 = Interleukin-6

IL-8 = Interleukin-8

IL-18= Interleukin-18

IL-13= Interleukin-13

LABA = Long-acting β -2 adrenoceptor agonist

LAMA = Long-acting muscarinic antagonist

LDL = Low-density lipoprotein

LRT = Lower respiratory tract

M catarrhalis = *Moraxella catarrhalis*

MGB = Minor groove binder

mL = milliliter

MM = Maintenance media

MMP = Matrix metallo-proteinase

MPO = Myeloperoxidase

NADPH = Nicotinamide adenine dinucleotide phosphate-oxidase

NF- κ B = Nuclear factor- κ B

NICE = National institute of health and clinical excellence

NK = Natural killer

NO = Nitric Oxide

NS = Non-structural proteins

PAF-R = Platelet activating factor - receptor

PBS = Phosphate-buffered saline

PCR = Polymerase chain reaction

PEF = Peak expiratory flow

PFU = Plaque forming units

PRO = Patient reported outcome

PR3 = Proteinase 3

qPCR = Quantitative polymerase chain reaction

ROS = Reactive oxygen species

RNA = Ribonucleic acid

RPM = Revolutions per minute

RSV = Respiratory syncytial virus

RT = Reverse transcription

RTP = Room Temperature

RT-PCR = Real-time PCR

SABA = Short-acting β -2 adrenoceptor agonist

SAMA = Short-acting muscarinic antagonist

SERPIN = Serine proteinase inhibitor

SNP = Single nucleotide polymorphism

S pneumoniae = *Streptococcus pneumoniae*

SVC= Slow vital capacity

TGF- β = Transforming growth factor- β

TNF- α = Tumour necrosis factor- α

μm = micrometer

μl = microliter

URT = Upper respiratory tract

VP 1-4 = Viral protein 1-4

Publications & Abstracts

Original article

Human rhinovirus infection during naturally occurring COPD exacerbations

SN. George, DS. Garcha, AJ. Mackay, ARC. Patel, R. Singh, RJ. Sapsford, GC. Donaldson and JA. Wedzicha (Síobhán Nicole George et al., 2014).

Oral presentations

Human rhinovirus load in stable COPD and at exacerbation (BTS 2011)

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Human rhinovirus infection and exacerbation frequency at COPD exacerbation (BTS 2013)

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The prevalence of clinically relevant micro-organisms in stable and exacerbated COPD using PCR techniques (ERS 2012)

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Time-course of rhinovirus and bacterial infection during COPD exacerbation recovery (BTS 2012)

SN. George, DS. Garcha, ARC. Patel, AJ. Mackay, R. Singh, RJ. Sapsford, GC. Donaldson and JA. Wedzicha (S. N. George et al., 2012).

Human rhinovirus infection and secondary bacterial infection in COPD exacerbations (ATS 2013)

SN. George, DS. Garcha, ARC. Patel, AJ. Mackay, R. Singh, RJ. Sapsford, GC. Donaldson and JA. Wedzicha (George et al., 2013).

Time-course of human rhinovirus infection and upper respiratory tract symptoms during COPD exacerbations (BTS 2014)

SN. George, SE. Brill, JP. Allinson, R. Singh, B. Kowlessar, RJ. Sapsford, GC. Donaldson and JA. Wedzicha (S. N. George et al., 2014)

CHAPTER 1. Introduction

1.1 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable but progressive airway disease which encompasses several heterogeneous phenotypes predominantly bronchitic and emphysematous. The chronic bronchitic component of COPD was first described in 1814 by British physician Charles Badham as a disabling disorder, using the word “catarrh” when referring to chronic cough and mucus secretions (Badham, 1814). Chronic bronchitis is defined clinically based on the presence of cough and sputum production for three months per year for two or more consecutive years (GOLD Report 2014; Scadding, 1959). The emphysematous aspect of COPD is characterised radiologically by the pathological appearance of large airspaces where there has been airway damage and destruction of alveolar walls (Horowitz et al., 2009). In 1829, French physician René Laennec discovered that certain patients with obstructed airways disease had hyper inflated lungs which did not empty efficiently (Laennec, 1829). This led the way to the development of the spirometer by John Hutchinson in 1846. In 1947, the spirometer was adapted to record values of forced vital capacity (FVC) which is the amount of air that can be forcibly exhaled from the lungs after taking the deepest breath possible (Yernault, 1997). It also records values for forced expiratory volume in one second (FEV_1) which is the volume of air exhaled during the first second of a forced expiratory manoeuvre. Both of these are now important measurements in COPD (Yernault, 1997).

The most widely accepted definition of COPD as described by the Global Initiative for COPD (GOLD) is “a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases” (GOLD Report 2014). The heterogeneous nature of COPD has led to the concept of multiple underlying phenotypes within COPD. By studying these phenotypes individually we can better our understanding and therefore treatment of this disease.

1.1.1 History and aetiology

COPD has been described in a number of different terms throughout the past. Several decades ago, a debate rose regarding the definition of this heterogeneous disease. In the 1950s and 1960s, two rival hypotheses emerged: the Dutch hypothesis and the British hypothesis. At the first Bronchitis symposium in the Netherlands in 1961, Orie put forward the hypothesis that various forms of airway obstruction such as asthma, chronic bronchitis and emphysema, should not be considered as separate diseases but different expressions of one disease entity (Orie, 1961) with both host and environmental factors playing a role in pathogenesis. This theory was later termed the “Dutch hypothesis” by Fletcher in 1969. In contrast, an alternative hypothesis termed the “British hypothesis” (Fletcher, 1976) suggested that a hereditary predisposition

towards developing allergy and bronchial hyperreactivity were considered to be important denominators of disease susceptibility.

The British hypothesis originated from the clinical observations that infectious exacerbations of COPD led to a continuing decline in lung function, were a risk factor for permanent decline in FEV₁ (Tager and Speizer, 1975) and that chronic bronchitis predisposed the lungs to subsequent infection resulting in airway damage leading to further progressive airflow limitation (Stuart-Harris et al., 1953). The proteinase (also referred to as protease)-antiproteinase theory is a combination of the Dutch and British hypotheses as broadly speaking the Dutch hypothesis is a genetic hypothesis and the British, an environmental one. The proteinase-antiproteinase hypothesis arises from the genetic defect in the levels of α_1 -anti-trypsin (AAT) in emphysema and also the observation that smoking increases the number of elastase-containing neutrophils. The British hypothesis was contested when Fletcher (Fletcher, 1976) and colleagues (Johnston et al., 1976) found no relationship between bronchial infections and FEV₁ decline and therefore the concept of two diseases, chronic bronchitis and emphysema, emerged.

1.1.2 Pathogenesis and Inflammation

In the healthy lung, the airways are defended by mechanisms such as mucociliary clearance, epithelial barriers and the recruitment of immune and inflammatory cells (Mizgerd, 2012). As inhalation is the most common route of entry to the lungs, they

are well adapted to eliminate foreign agents which may cause infection or irritation within the airways. Alveolar macrophages ensure pathogens do not persist in the airways and cause harm; they are supported by other immune cells of the innate immune system such as neutrophils (Barnes, 2008). The innate defence in the airways is designed to remove foreign and potentially harmful particles and resolve inflammation, both of which are beneficial responses in the case of healthy individuals. In patients with COPD however, the actions of the immune system can result in an abnormal and exaggerated inflammatory response that can progress to pathology within the lung (Baines et al., 2011).

Cigarette smoke is likely to account for approximately 80-90% of COPD cases in the developed world (Sethi and Rochester, 2000). In COPD patients, cigarette smoke interferes with the innate defences by increasing mucus production, reducing mucociliary clearance, disrupting the epithelial cell barrier and stimulating the migration of immune cells such as neutrophils, macrophages and monocytes, natural killer (NK) cells, dendritic cells, CD4⁺, CD8⁺ and B-cell lymphocytes to the area of tissue damage (Hogg et al., 2004). The production of these cells in turn leads to an abundance of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, neutrophil elastase, myeloperoxidase and matrix metalloproteinases (MMPs) (Yamamoto et al., 1997) within the airway. It is the abnormal inflammatory response to the inhalation of noxious particles found in COPD patients that leads directly, and indirectly, to airway and parenchymal lung damage (Hogg et al., 2004).

Cigarette smoking leads to airway inflammation in all those that smoke, with or without COPD (Yanbaeva et al., 2007). However in patients with COPD, the pathological abnormalities persist even after the removal of the noxious stimuli, for example following smoking cessation, and from this it has been suggested that certain individuals are more susceptible to the development of COPD (Cosio et al., 2009). The characteristic irreversible airflow limitation of COPD is caused by a thickening of the airway wall due to fibrosis and inflammation, and also hypersecretion of mucus in the lumen of the airway (Figure 1.1).

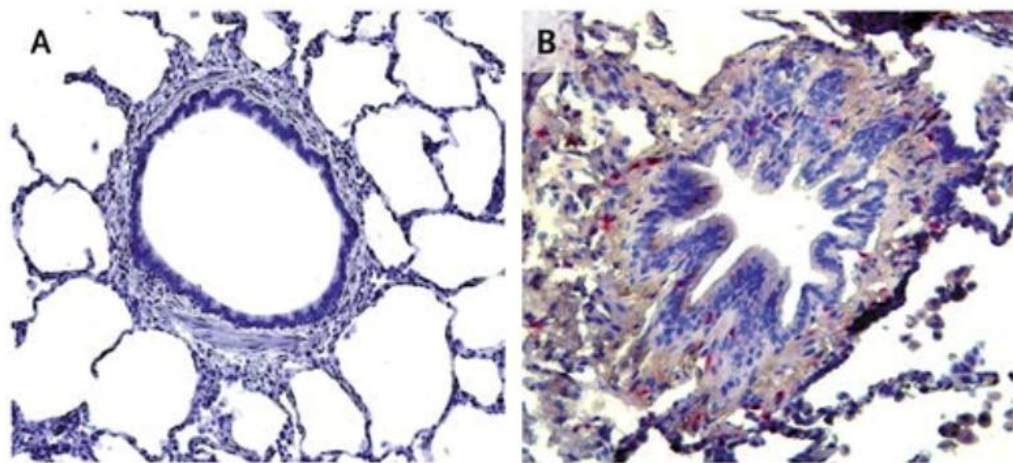


Figure 1.1: In the specimen of the small airways (membranous bronchioles) from the healthy lung of a non-smoker (Panel A), the airway walls are thin, and intact alveoli are attached along its circumference. In a comparable specimen from the lung of a smoker with COPD (Panel B), the diameter of the airway is narrowed, the airway wall is thickened, and many of the alveolar attachments are broken. CD8+ T lymphocytes (in red) infiltrate the airway wall in the specimen from the smoker with COPD (Panel B) but not in the specimen from the non-smoker (Panel A) (immunostaining with antihuman CD8; counterstained with haematoxylin) (Cosio et al., 2009).

Airway epithelial cells and macrophages activate transcription factors such as nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$) which is present in the cytosol in an inactive form. NF- $\kappa\beta$ regulates the synthesis of many of the cytokines and chemokines that are secreted in COPD such as IL-8, TNF- α and nitric oxide (NO) which play a role in the amplification of airway inflammation (Caramori et al., 2003). Bronchial airway biopsies have shown an increase in the number of neutrophils in the airway lumen of COPD patients but there is no evidence for the activation of mast cells (Hogg, 2004). The combination of epithelial-cell growth factors and inhaled cigarette smoke can cause chronic irritation within the airways which can lead to epithelial cells showing pseudostratification (there appears to be more than one layer of cells when in fact there is just one) (Barnes, 2008). Mucus hyperplasia and increased mucin expression have been found in COPD patient biopsies (Caramori et al., 2004). COPD patient lungs have also been shown to have a higher production of elastolytic enzymes such as neutrophil elastase and several MMPs. Furthermore a reduction in the levels of antiproteinases such as AAT (*section 1.1.4.1*) has been shown in rare cases of emphysema caused by a genetic deficiency (Tuder et al., 2006). Macrophages are derived from circulating monocytes which respond to chemoattractants and migrate to the lungs. Macrophages are found in high numbers in the lungs of COPD patients and it is believed that they may coordinate the inflammation of COPD by the release of chemokines that attract monocytes, T cells, proteinases and neutrophils (Figure 1.2). Neutrophils are found in increasing numbers in the sputum of COPD patients compared to healthy smokers and non-smokers, which has been shown to correlate with disease severity (Keatings et al., 1996). This neutrophilia is linked to an increase in the production of chemokines such

as CXCL1/GRO α and IL-8. T helper 1 (Th1) CD4⁺ T cells are attracted to the lungs by CXCR3 ligands such as CXCL0, CXCL10 (also known as IP-10) and CXCL12 (Costa et al., 2008; Saetta et al., 2002) that are released from interferon- γ (IFN- γ). T helper 2 (Th2) cells have also been shown to be significantly higher in the bronchoalveolar lavage fluid of patients with COPD compared to healthy controls; $p=0.01$ (Barczyk et al., 2006). The immunological responses in COPD patients are still not fully understood but it has been proposed that immunoglobulins secreted by T cells, and their regulation, may be activated by either bacterial or viral antigens from chronic bacterial colonisation or latent viral infection in the patient's airways. Further hypotheses suggest that there may be an autoimmune component in COPD in which new antigenic epitopes are developed as a result of the tissue damage caused by cigarette smoking, oxidative stress or chronic bacterial infection (Agustí et al., 2003).

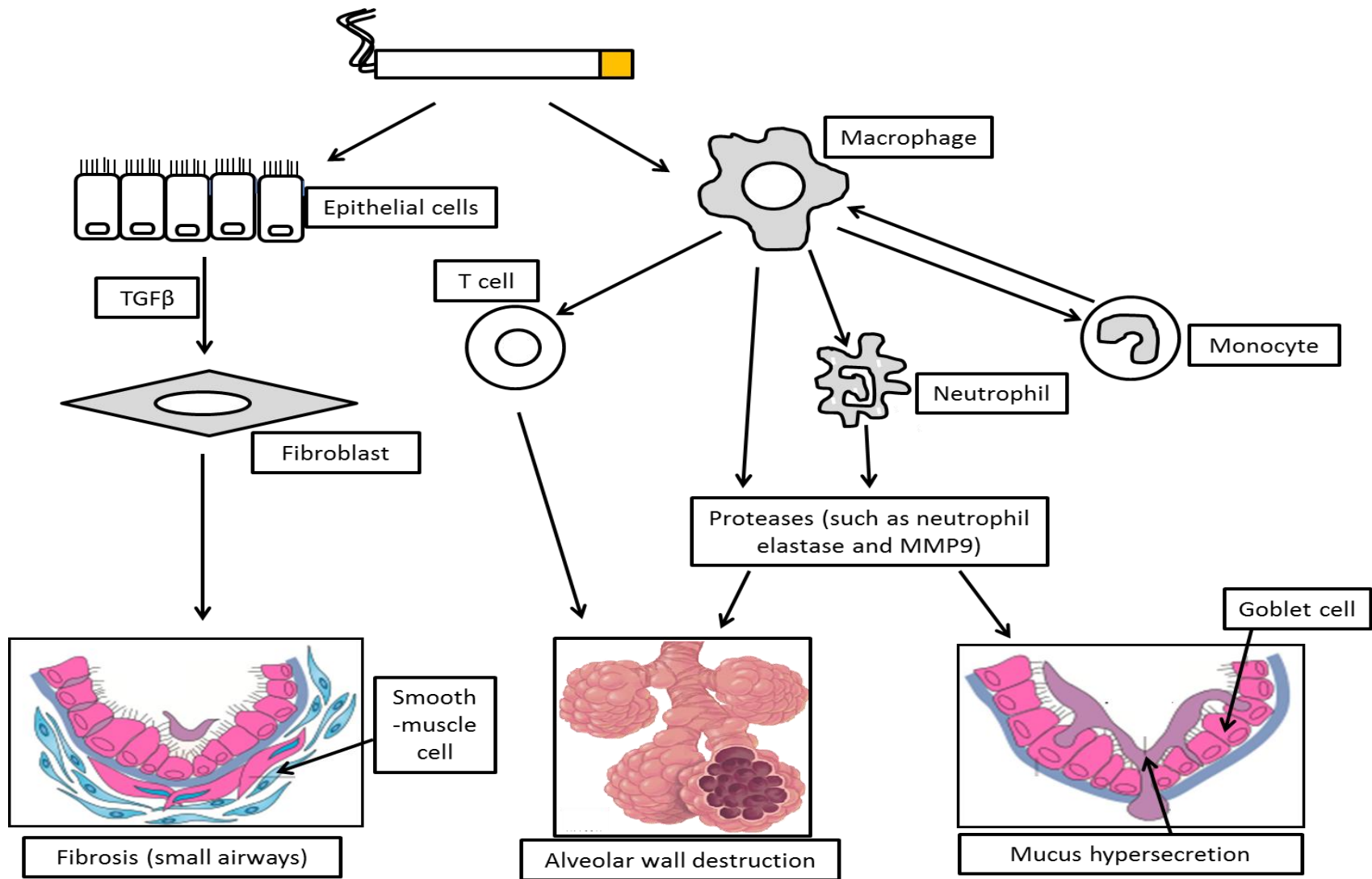


Figure 1.2: Summary of the inflammatory and immune cells involved in COPD (Barnes, 2008).

Until recently the focus of inflammation and inflammatory changes with regard to COPD has been in the airways (Sinden and Stockley, 2010). However it is becoming more apparent that systemic inflammation plays a role in the disease itself and in relation to secondary inflammation.

1.1.2.1 Systemic Inflammation

The inflammatory response observed in the lungs of COPD patients shows evidence of innate and acquired immune activation (Cosio et al., 2009). The accumulation of inflammatory components in these patients contributes not only to lung damage and injury but to further immune activation. Systemic inflammation has been reported in patients with COPD, especially those with increasing severity in the stable state and has been measured by examining changes in circulating cytokines such as IL-6 and TNF- α (Yanbaeva et al., 2007), chemokines including IL-8 and leptin (Takabatake et al., 1999), and other proteins such as C-reactive protein (CRP) (Broekhuizen et al., 2006) and fibrinogen (Donaldson et al., 2005). Systemic inflammation been shown to be associated with an accelerated decline in lung function and is higher during COPD exacerbations (Donaldson et al., 2005). Smoking may be a contributor to systemic inflammation (Fröhlich et al., 2003) and it has been shown that COPD patients have greater levels of systemic inflammation compared to healthy controls (Schols et al., 1996). It is unclear whether the systemic inflammation seen during COPD is due to systemic marker over-spill from the lungs or whether systemic inflammation just

reflects the generalised proinflammatory state (Barnes and Celli, 2009; Sinden and Stockley, 2010). Furthermore, it has been suggested that systemic inflammation could be a result of a comorbid disease which in turn affects the lungs (Sinden and Stockley, 2010).

1.1.3 Oxidant/antioxidant imbalance

Cigarette smoke contains an estimated 4700 different chemical compounds and approximately 10^{15} - 10^{17} oxidants such as free radicals, nitric oxide and nitrogen dioxide per puff (Church and Pryor, 1985). As well as this, cigarette smoke stimulates tissues in the lungs to produce oxidants (Barreiro et al., 2010) and both of these factors lead to high levels of oxidants being present resulting in uncontrolled tissue destruction. In the body of healthy individuals, oxidants that are produced are counteracted by antioxidants such as glutathione and vitamin C. Antioxidants work as reducing agents by reducing the oxidants and therefore removing oxidative stress (MacNee, 2000). It is believed that an imbalance between oxidants and antioxidants plays a role in the pathogenesis of COPD and that COPD patients have an increased oxidative burden. This can lead to many of the pathogenic processes that are associated with COPD including increased inflammation and direct injury to the lung (MacNee, 2005). It has been shown that oxidative stress is higher in the lungs of patients with COPD compared to healthy smoking subjects (MacNee, 2000); this is not surprising as cigarette smoke

introduces so many oxidants. However, oxidative stress was also found to be higher in COPD patients compared to smokers without COPD (MacNee, 2000) suggesting that there are other mechanisms contributing to oxidative stress in COPD patients, in addition to smoke. Chronic airway inflammation is a major secretor of reactive oxygen species (ROS) as both neutrophils and macrophages, which migrate in increased numbers into the lungs of COPD patients (Barnes, 2004), can generate ROS via the NADPH oxidase system (Rahman and MacNee, 1996). ROS are chemically reactive molecules containing oxygen and are a natural by-product of the normal metabolism of oxygen (Bayr, 2005). When ROS levels increase dramatically, damage to cell structures (oxidative stress) can occur (MacNee, 2001). As the iron content the alveolar macrophages of smokers is higher than in non-smokers (MacNee, 2001) the resultant increased free iron in the airspaces of smokers can simulate the stimulation of ROS (Thompson et al., 1991). Oxidants present in cigarette smoke can also stimulate alveolar macrophages to produce ROS and to release mediators that attract immune cells such as neutrophils which in turn drive further increases production of ROS.

1.1.4 Causation

The predominant aetiological agent of COPD is cigarette smoking with approximately one in five smokers developing COPD. However, exposure to other environmental factors such as air pollutants, noxious particles or gases, biomass fuels, poverty,

nutritional factors and infection have also been found to lead to the development of COPD (Anthonisen et al., 2005). Similarly, the use of or exposure to biomass fuels has been linked to COPD development particularly in females, occurring more commonly in developing countries where this type of fuel is more readily used in the home for cooking and heating where ventilation is often very poor or absent (Liu et al., 2007).

1.1.4.1 Genetics

Although at least 85% of COPD patients develop COPD from tobacco smoking, a significant minority of cases are thought to be due to other aetiological factors (Babusyte et al., 2007). More recently, increasing attention has been given to the role of genetics in pre-disposing certain individuals to COPD. For example AAT deficiency is a well-established, but rare, genetic cause of COPD development with approximately 1-2% of COPD patients having AAT as the principle factor (DeMeo and Silverman, 2004). AAT is a proteinase inhibitor that is coded for by the *SERPINA1* gene (Brantly et al., 1988) found on the proteinase inhibitor locus. It is synthesised by hepatocytes in the liver and functions as a serine proteinase inhibitor or serpin that provides essential protection to the lung tissue against action from proteolytic enzymes such as neutrophil elastase, which breaks down elastin in the lungs, and proteinase 3 (PR3) (Sinden and Stockley, 2013). The active site of AAT binds to elastase which results in its permanent inactivation (DeMeo and Silverman, 2004). Four main AAT phenotypes exist in the general population, identified by the differences in speed of the proteins on gel

electrophoresis. These are normal alleles, deficiency alleles, null alleles and dysfunctional alleles. Those that inherit null alleles have AAT protein levels that are not detectable in the serum which means they are particularly at risk of acquiring emphysema (Luisetti and Seersholm, 2004). Normal alleles are the most prominent phenotype and are found in approximately 95% of the Caucasian population (DeMeo and Silverman, 2004). Neutrophilia, frequently seen in the lungs of COPD patients secrete neutrophil elastase resulting in the loss of alveolar structure (Mordwinkin and Louie, 2007). In healthy individuals, AAT would bind to the neutrophil elastase, inhibiting it, and therefore preventing alveolar damage and degradation. In AAT deficient subjects, elastase cannot be countered efficiently resulting in the damage of lung tissue and the development of emphysema (Abboud et al., 2005). Although the role of neutrophil elastase in the pathogenesis of emphysema in AAT subjects is well investigated, there may be other proteinases such as PR3 that are also relevant. PR3 is found in the azurophil granules of neutrophils and has also been shown to cause emphysema in animal models (Kao et al., 1988). A study by Sinden and Stockley in 2013 looking at stable COPD patients with either AAT deficiency or with “usual” COPD with chronic bronchitis showed that PR3 was detected in most sputum samples and was higher than the neutrophil elastase activity in both groups (Sinden and Stockley, 2013). It was also found that PR3 levels were significantly higher at exacerbation compared to the stable state ($p=0.037$) suggesting that PR3 may have a role in the pathophysiology of COPD in these groups (Sinden and Stockley, 2013).

Genetic susceptibility studies have demonstrated that TNF- α and interleukin-13 (IL-13) promoter polymorphisms are significantly associated with the presence of smoking-related COPD. A study involving non-COPD long-term smokers as controls ($FEV_1/FVC \geq 70\%$) and long-term smokers with COPD ($FEV_1/FVC < 70\%$) showed that single nucleotide polymorphisms encoded by the *ADAM33* gene are associated with COPD in long-term smokers (Sadeghnejad et al., 2009) suggesting there may be a genetic mechanism that leads to some long-term smokers developing COPD whereas others do not.

1.1.5 Diagnosis

The diagnosis of COPD is confirmed clinically based on three factors. Firstly, patients must have a history of chronic lower respiratory tract symptoms such as dyspnoea, wheeze, cough and sputum production which begin or worsen in middle to later life and continue to do so. Secondly there should be a history of sufficient exposure to noxious particles or gases. If there is not, there should be evidence of increased susceptibility such as strong family history of COPD or AAT (Patel and Hurst, 2011). Thirdly, confirmatory post-bronchodilator spirometry testing is required following a set of recommendations defined by GOLD (GOLD Report 2014) (Table 1.1 below). The test requires the patient to take a large breath and then breathe out as hard and fast as they can into the spirometer. The amount of air the patient exhales in the first second

of the breath is called the forced expired volume in one second (FEV_1). The forced vital capacity (FVC), which quantifies the volume of expired air from the lungs, is also measured. COPD is diagnosed if the FEV_1 : FVC ratio is <0.7 . The joint guidelines set by the European Respiratory Society (ERS) and American Thoracic society (ATS) state that COPD diagnosis is confirmed if the ratio of FEV_1 : SVC (slow vital capacity) is within the lower limit of detection of the normal distribution, rather than at a fixed value of 0.7 (Shirtcliffe et al., 2007). In the London COPD cohort, the fixed ratio of 0.7 rather than the lower limit of normal is used.

Grade/Severity of COPD	Spirometric cut off
I Mild	$FEV_1/FVC < 0.70$ $FEV_1 \geq 80\%$ predicted
II Moderate	$FEV_1/FVC < 0.70$ $50\% \leq FEV_1 < 80\%$ predicted
III Severe	$FEV_1/FVC < 0.70$ $30\% \leq FEV_1 < 50\%$ predicted
IV Very Severe	$FEV_1/FVC < 0.70$ $FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted plus chronic respiratory failure

Table 1.1: Spirometric classification of COPD severity based on the FEV_1 from the GOLD guidelines (GOLD Report 2014).

1.1.6 Epidemiology and Impact

Currently, COPD is the third most common cause of mortality (Lozano et al., 2012) and has a significant impact in terms of both morbidity and mortality. Between 1980 and 2000, recorded deaths due to COPD rose 14% in men and 185% in women (Pauwels et al., 2001). Biological differences between men and woman may account for the difference in this percentage increase. Women have smaller lungs and larger airways than men of comparable size (Mead, 1980). Lung size may be related to decline in lung function and airway size may influence smoke distribution which could mean a mechanical factor is responsible for an increased sensitivity of females to cigarette smoke. Alternatively hormonal factors have been suggested to play a role; studies using animal models have shown that female rats have a greater increase in both the number and size of goblet cells on exposure to cigarette smoke compared to male rats (Hayashi et al., 1978). Other factors such as differences in the inflammatory response and airway responsiveness have also been suggested as well as the likelihood of women being more regularly exposed to biomass fuel in developing countries compared to men (Liu et al., 2007). Furthermore, this difference could partly be attributed to the changes in the numbers of young women smoking in modern times.

COPD has become a major global health burden resulting in 30, 000 deaths annually in the UK alone (Wildman et al., 2007). As the predominant risk factor associated with the

development of COPD is tobacco smoking, it is considerably more cost-effective to educate and encourage people to stop smoking, prior to the development of COPD rather than to treat them clinically after COPD has been diagnosed. However in patients who have stopped smoking therapy is often required to ease symptoms.

1.1.7 Therapy in stable COPD

Although there is no cure for COPD, there are a number of therapies available to alleviate symptoms and improve disease indices including lung function and exacerbation frequency. The most effective and successful treatment for COPD is smoking cessation; an aggressive smoking intervention programme showed a significant reduction in the age-related decline in FEV₁ in middle-aged smokers with mild airway obstruction (Anthonisen et al., 2002). In 1977 Fletcher and Peto reported that smoking cessation in a healthy smoker will make little difference to their FEV₁ if their lungs are not being dramatically affected by the smoke (Figure 1.3). However in patients with airway obstruction, smoking cessation may dramatically reduce the rate of FEV₁ decline such that the subsequent loss of lung function will slow to the normal rate (Fletcher and Peto, 1977). However whether or not patients continue to smoke, some degree of pharmacotherapy is usually required once established COPD is present.

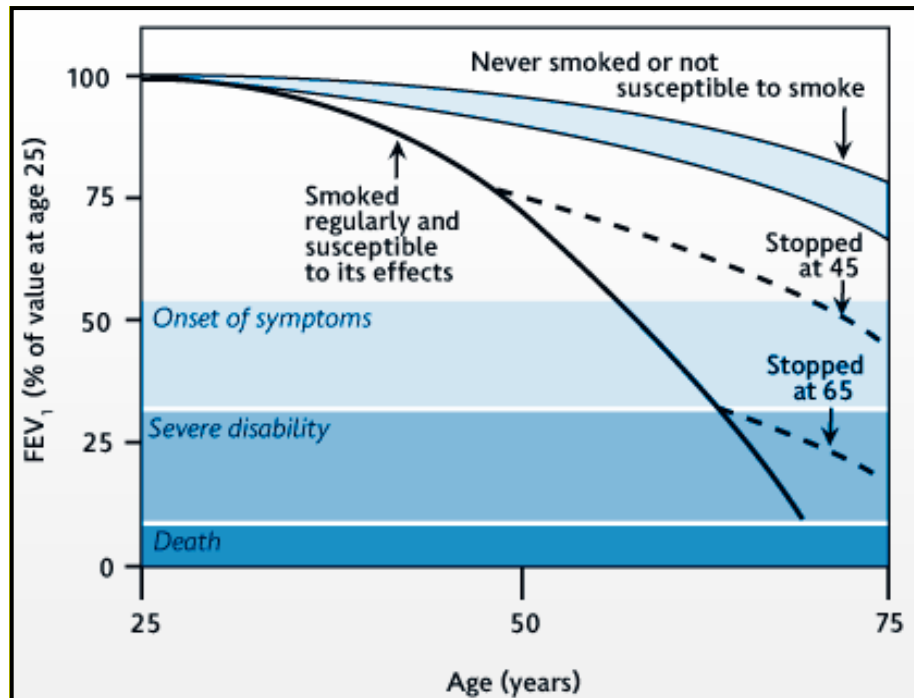


Figure 1.3: Risks for various men if they smoke: differences between these lines illustrate the effects that smoking, and stopping smoking, can have on FEV₁ of men who are liable to develop COPD if smoking. Death, the underlying cause of which is irreversible chronic obstructive lung disease, whether the immediate cause of death is respiratory failure, pneumonia, cor pulmonale, or aggravation of other heart disease by respiratory insufficiency. Although this shows rate of loss of FEV₁ for one particular susceptible smoker, other susceptible smokers will have different rates of loss, thus reaching “disability” at different ages (Fletcher and Peto, 1977).

1.1.7.1 Bronchodilators

Bronchodilators are a common treatment in stable COPD for the relief of symptoms, mainly dyspnoea (Cazzola and Matera, 2014). They are delivered by inhalation and

work by altering airway smooth muscle tone. Widening of the airways results in improved expiratory flow and lung emptying, reduced hyperinflation and improved exercise performance. Bronchodilators are used either as needed when symptoms arise (as a 'reliever') or regularly to prevent symptoms (as a 'preventer'). There are two main types of bronchodilator, beta₂-agonists and anticholinergics and their main function is to reduce symptoms and increase exercise tolerance.

a. Beta₂-agonists

Beta₂-agonists are the most common class of bronchodilator. Their main mode of action is to stimulate airway beta₂-adrenergic receptors and increase levels of cyclic AMP. This reduces functional antagonism to bronchoconstriction and results in smooth muscle relaxation and airway dilation (GOLD Report 2014). Short-acting beta₂-agonists (SABAs) such as salbutamol and terbutaline have a rapid onset but short duration of action (4-6 hours) (van Schayck et al., 1991) and are therefore usually taken as relievers. Long-acting beta₂-agonists (LABAs) such as salmeterol, formoterol and indacaterol produce effects after approximately 20 minutes but last for 12-24 hours (Decramer et al., 2013), and are therefore usually taken regularly as preventers. Regular use has been shown to improve FEV₁ and symptoms (Skillrud et al., 1986); in particular, both formoterol and salmeterol significantly improve FEV₁ (Boyd et al., 1997; Cazzola et al., 1995), dyspnoea (Ulrik, 1995), quality of life (Rossi et al., 2002) and exacerbation frequency (Rossi et al., 2002). Indacaterol lasts for 24 hours with a larger

bronchodilator effect that has been shown to be significantly greater than formoterol or salmeterol in terms of health status, exacerbation rate and symptomatic improvements (Rossi and Polese, 2013). Side effects of beta₂-agonists include sinus tachycardia, tremor, hypocalcaemia and rarely tachyphylaxis and occasional hypoxia (Khoukaz and Gross, 1999; Polverino et al., 2007; Uren et al., 1993).

b. Anticholinergics

Anticholinergics are muscarinic antagonists, another type of bronchodilator, which work by blocking the effect of acetylcholine on muscarinic receptors and therefore preventing bronchial constriction (Cazzola et al., 2013). The short-acting muscarinic antagonist (SAMA) ipratropium, targets M2 and M3 receptors and modifies signal transmission at the pre-ganglionic junction. Like SABAs, it can be inhaled or nebulised and has a rapid onset of action (15 minutes) suitable for use as reliever therapy, lasting up to 6 hours. Long-acting muscarinic antagonists (LAMA) such as tiotropium block M1 and M3 receptors (Disse et al., 1999) and the long duration of action over 24 hours allows use as a once-daily preventer (van Noord et al., 2000). New long-acting LAMAs are mainly M3 receptor blockers with less M1 and minimal M2 activity. Tiotropium has been shown to reduce exacerbations and related hospitalisations (Tashkin et al., 2008), improve symptoms (Vincken et al., 2002), health status (Casaburi et al., 2002) and the effectiveness of pulmonary rehabilitation (Tashkin et al., 2008). Side effects of anticholinergics include dryness of the mouth and glaucoma. A 2010 meta-analysis of

safety data from large published trials (Llor et al., 2012) raised concerns regarding an excess of cardiovascular mortality using the Respimat, a soft-mist tiotropium delivery device, in patients with a history of arrhythmia. However, a large and well-conducted trial, involving 17,135 patients and adequately powered to detect differences in mortality between tiotropium dose and delivery methods, has recently found this not to be the case (Wise et al., 2013).

1.1.7.2 Inhaled corticosteroids (ICS)

ICS are the most effective anti-inflammatory therapy for many chronic inflammatory diseases such as asthma; however the long-term effect of ICS in COPD is limited (Barnes, 2006). Regular treatment has been shown to improve symptoms, lung function, quality of life and reduce exacerbation frequency (Spencer et al., 2004) in patients with an FEV₁ <60% predicted. In asthma the efficacy and side effects of the drugs are dependent on the dose but this is not necessarily the case for COPD patients (Bateman et al., 2008). Side effects of ICS are mainly local and include oral fungal infections. The TORCH trial first highlighted an increased rate of pneumonia in COPD patients taking ICS compared to those on alternative treatments (Calverley et al., 2011). This finding has been subsequently confirmed in the inspire study (Wedzicha et al., 2008), and appears to be dose-dependent (Suisse et al., 2013). Garcha and colleagues showed that higher ICS dose was significantly correlated to higher airway bacterial load in the stable state (Garcha et al., 2012). The risk-benefit ratio of ICS in

COPD is therefore not as clear-cut as previously thought, although they remain important treatments in COPD.

1.1.7.3 Combination therapy

The combination of ICS and long-acting bronchodilator therapy (beta₂-agonist), usually in a single inhaler (e.g. fluticasone & salmeterol, budesonide & formoterol, beclomethasone & formoterol) has been shown to be more effective in improving lung function and health status than either treatment given individually (Wedzicha et al., 2013). Combination therapy is also associated with reducing exacerbations in moderate and severe COPD patients (Wedzicha et al., 2013); however, as stated previously, there is an increase in pneumonia (Crim et al., 2009). The addition of tiotropium to the combination therapy improves quality of life, lung function and may reduce exacerbations however more studies into triple therapy are required (Perng et al., 2006).

1.1.7.4 Vaccinations

Vaccines that contain killed or live inactive pathogens are most effective in elderly COPD patients. Influenza vaccinations can reduce lower respiratory tract infections, hospitalisation and death (Nichol et al., 1994; Wongsurakiat et al., 2003). The vaccine is adjusted yearly to ensure appropriate protection against the most prevalent influenza

strains at that time, and the vaccine should be given yearly (Woodhead et al., 2005). Influenza vaccination has been shown to reduce the incidence of community acquired pneumonia in elderly patients with severe COPD (Alfageme et al., 2006). The incidence of pneumococcal infections is highest at the extremes of age, and therefore pneumococcal polysaccharide vaccine is separately recommended for all COPD patients over 65 years of age and other patients with co-morbidities such as cardiac disease (Jackson et al., 2003).

1.1.8 Comorbidities

COPD has many comorbidities associated with it which can lead to exacerbations, hospital admissions, more intense symptoms and even mortality (Patel and Hurst, 2011). The most common comorbidities include psychological conditions, musculoskeletal and cardiovascular problems and COPD patients are more likely to die from a comorbid disease than from COPD itself (Patel and Hurst, 2011). In a large, randomised control trial of inhaled combined fluticasone/salmeterol in 2007, causes of death were recorded. All 6184 patients had an FEV₁ of <60% and well characterised COPD. Of the 911 deaths, only 40% were definitely or probably related to COPD with 50% unrelated to COPD and 9% unknown (McGarvey et al., 2007). This illustrates the importance of comorbidities in COPD and that the appropriate management of them may have large mortality benefits. Some comorbidities occur coincidentally due to their

occurrence in later life such as osteoporosis and Alzheimer's disease. Others have shared risk factors such as smoking but have not been shown to be any more common in patients with COPD such as oral and laryngeal cancers and smoking-related bladder cancer. However there are many-comorbidities that have increased incidence when shared risk factors are accounted for. These include cardiovascular disease such as congestive heart failure, myocardial infarction, stroke (Donaldson et al., 2010) and ischemic heart disease (Patel et al., 2012). Similarly, gastro-oesophageal reflux disease (García Rodríguez et al., 2008), depression and anxiety (Quint et al., 2008), skeletal muscle dysfunction (Swallow et al., 2007) and diabetes (Areias et al., 2014) have all been shown to be related to COPD. Table 1.2 below shows the prevalence of individual comorbidities.

Comorbidity	Prevalence in COPD (%)
Osteoporosis/osteopenia	50-70
Hypertension	40-60
Gastro-oesophageal reflux disease	30-60
Skeletal muscle dysfunction	32
Depression	25
Ischemic heart disease	10-23
Previous myocardial infarction	4-32
Anaemia	17
Diabetes	12-13
Previous stroke	10-14
Arrhythmia	6-14
Chronic renal failure	6-11
Congestive heart failure	5-7
Obstructive sleep apnoea	1-4

Table 1.2: The prevalence of comorbidities in COPD (Patel and Hurst, 2011).

The majority of COPD patients have at least one important comorbidity and these extra-pulmonary comorbidities, which often have pathophysiological links, can have a

large impact on morbidity and mortality, increased risk of hospitalisation and greater healthcare costs (Patel and Hurst, 2011).

1.2 Acute Exacerbation of COPD

1.2.1 Exacerbations

COPD patients can suffer episodes of acute worsening of respiratory symptoms associated with variable degrees of physiological deterioration known as acute exacerbations of COPD (AE-COPD). An exacerbation is defined in the GOLD guidelines as “an event in the natural course of the disease characterised by a change in the patient’s baseline dyspnoea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, and may warrant a change in regular medication in a patient with underlying COPD” (GOLD Report 2014). In practice and in the London COPD cohort, an exacerbation is defined as a worsening of two or more symptoms for two or more consecutive days with at least one symptom being a major symptom. Major symptoms are dyspnoea, increased sputum purulence and increased sputum volume and the minor symptoms are cough, wheeze, sore throat and cold symptoms. The impact of an exacerbation may take a patient several weeks to return to the stable state and can lead to irreversible deterioration of lung function (Seemungal et al., 2000).

1.2.2 Frequent and non-frequent exacerbators

The impact of COPD varies from patient to patient including how frequently a patient suffers from an exacerbation. If a patient in the London COPD cohort has less than two exacerbations per year, they are defined as an infrequent exacerbator using the London COPD cohort definition. If, however, they suffer two or more exacerbations per year, they are considered a frequent exacerbator. Frequent exacerbators experience a poorer quality of life (Seemungal et al., 1998), greater airway inflammation (Bhowmik et al., 2000) and faster lung decline (Donaldson et al., 2002) compared to infrequent exacerbators. A study by Hurst and colleagues from the ECLIPSE observational study in 2010 tested the hypothesis that there is a frequent exacerbator phenotype of COPD in 2138 patients with COPD. The study reported that exacerbations become more frequent and more severe as the severity of COPD increases; exacerbation rates for patients with moderate disease (GOLD stage 2) were 0.85 per person per year compared to 1.34 in those with severe disease (GOLD stage 3) and 2.00 with very severe disease (GOLD stage 4) (Hurst et al., 2010). They also reported that the major determinant of frequent exacerbations in all GOLD stages of COPD severity was a history of exacerbations and that the frequent exacerbator phenotype can be identified on the basis of a history of exacerbations. Among patients who had frequent exacerbations in year 1 and 2, 71% went on to have frequent exacerbations in year 3

whereas 74% of patients who had no exacerbations in year 1 and 2 also had no exacerbations in year 3 (Hurst et al., 2010).

1.2.3 Treatment for exacerbations

The three classes of medication most commonly used for acute exacerbations of COPD are antibiotics, oral steroids and bronchodilators, singly or in combination. Viral infection is often indicated by the presence of cold symptoms whereas increases in sputum volume or purulence suggest bacterial overgrowth and a possible benefit from antibiotic therapy (Anthonisen et al., 1987).

1.2.3.1 Antibiotics

Although the infectious agents involved in COPD exacerbations can be viral or bacterial, evidence underlying the use of antibiotics for exacerbations is incomplete. There is strong evidence supporting the use of antibiotics in severe exacerbations requiring hospitalisation (Vollenweider et al., 2012) or when patients have clinical signs of a bacterial infection such as increase in sputum purulence (Stockley et al., 2000). In addition, antibiotics have been shown to reduce the risk of short-term mortality in moderate or severe COPD patients with typical exacerbation symptoms (Llor et al., 2012). A typical treatment course of antibiotics for COPD exacerbations is 7-10 days with a second course given if symptoms do not improve (GOLD Report 2014). Antibiotic

selection should be tailored according to patient tolerance and the airway pathogens that are likely to be prevalent. Antibiotic classes have good activity against the spectrum of bacteria usually found in the airway include β -lactams, fluoroquinolones, macrolides and tetracyclines. The most commonly prescribed antibiotics in the London COPD cohort are amoxicillin and co-amoxiclav (combination of amoxicillin and clavulanic acid) which inhibit synthesis of the peptidoglycan cell wall in replicating bacteria (Tomasz, 1979). Macrolide antibiotics such as erythromycin, clarithromycin and azithromycin are important as they have also been shown to have anti-inflammatory properties in addition to their anti-bacterial properties, such as reducing IL-8 production and neutrophil migration and/or function (Cameron et al., 2012). This has led to a renewed interest in using macrolides prophylactically to reduce exacerbation frequency, and there is a growing body of evidence for this (Albert et al., 2011; Seemungal et al., 2008). There are concerns, however, regarding a possible increase in cardiovascular events (Ray et al., 2012), hearing loss (Albert et al., 2011) and the induction of bacterial resistance (Serisier, 2013). At present therefore, routine usage of prophylactic antibiotics is not recommended.

1.2.3.2 Oral corticosteroids

Oral corticosteroids in COPD exacerbations have been shown to shorten recovery time, improve lung function, reduce the risk of recurrent exacerbation and reduce the length of hospital stay (Davies et al., 1999; Maltais et al., 2002). There is insufficient data to

confirm the optimal duration of oral corticosteroid treatment at COPD exacerbation but 7-10 days is usual in clinical practice (Walters et al., 2005). Short-term side effects of oral corticosteroids include mood changes, increased appetite and impaired diabetic control. Longer-term side effects, which usually only occur if the duration of therapy extends into months or patients are prescribed repeated short courses, include adrenal insufficiency, peripheral muscle weakness, glucose intolerance, osteoporosis, easy bruising and weight gain.

In a study comparing groups of patients given either budesonide, prednisolone or placebo to treat acute exacerbations of COPD, it was found that budesonide and prednisolone significantly improved FEV₁ compared to the placebo group but there was no difference between the two treatment groups (Maltais et al., 2002). However a significant number of subjects given prednisolone became hyperglycaemic (Maltais et al., 2002). Short courses of oral steroids are generally well tolerated however, and remain one of the mainstays of treatment for COPD exacerbation. A study in 2013 showed how short courses of oral steroids for example 5 days, can be as efficient as longer courses for most COPD exacerbations (Sin and Park, 2013).

1.2.3.3 Bronchodilators

Patients may increase their bronchodilator therapy (also known as reliever medication) during COPD exacerbation, and higher doses may be administered by nebulisation.

SABAs with or without SAMAs are usually the preferred bronchodilators for treatment of symptoms of exacerbations (GOLD Report 2014) in the form of salbutamol in response to increased symptoms such as breathlessness, cough, wheeze, chest tightness.

1.2.4 Micro-organisms and COPD exacerbations

Respiratory illnesses can develop in the upper and lower respiratory tract and are predominantly due to infection by bacteria, viruses or fungi. In healthy individuals, these infections are naturally resolved by the immune system leading to a controlled inflammatory response. In COPD patients however, infections can result in an exaggerated increase in inflammation on an already inflamed COPD airway, causing greater inflammation which may lead to an exacerbation (Wedzicha and Seemungal, 2007) (Figure 1.4). Evidence suggests that 50-70% of COPD exacerbations have an infectious aetiology (Sapey and Stockley, 2006) with viruses being a key trigger. A study by Papi and colleagues found that 78% of patients with severe exacerbations requiring hospitalisation were positive for a viral and/or a bacterial infection; 48.4% positive for a virus and 54.7% for bacteria compared to those in the stable state where only 6.2% were positive for a virus and 37.5% for bacteria (Papi et al., 2006).

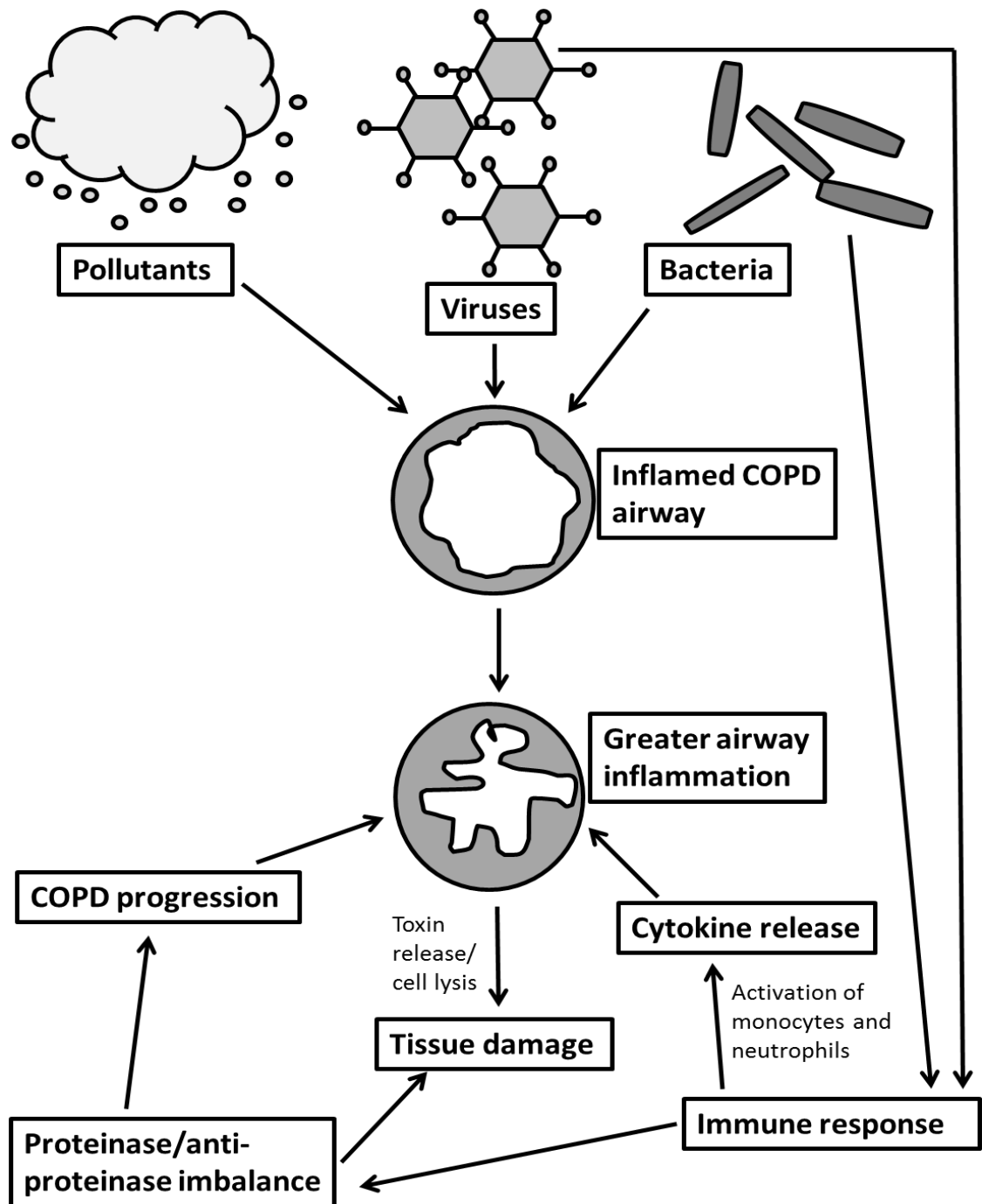


Figure 1.4: Triggers of COPD exacerbations and the vicious circle hypothesis in COPD adapted from Sethi and Murphy, 2001; Wedzicha and Seemungal, 2007.

1.2.4.1 Viruses at COPD exacerbation

As previously discussed, exacerbations of COPD are associated with airway and systemic inflammation that can be triggered by aetiological factors such as bacterial or viral infection. Bacterial infections are considered to be a significant cause of COPD exacerbations (Garcha et al., 2012) which is highlighted by the large scale use of antibiotics given at exacerbation. Their exact role remains contentious as bacteria are only found in approximately half of exacerbations but are also found in patients who are stable (Mallia and Johnston, 2006). There has been increasing evidence that respiratory viruses cause COPD exacerbations; the prevalence of viruses has been shown to be higher at exacerbation compared to in the stable state with a respiratory virus being detected in 48% of exacerbations (Papi et al., 2006).

An association between the presence of respiratory viruses and greater airway inflammation has been reported (Wilkinson et al., 2006), and epidemiological data has shown that many patients report cold symptoms during their exacerbations (Hurst et al., 2005; Jenkins et al., 2012). Viral infections are associated with increased severity and frequency of COPD exacerbations (Seemungal et al., 2000) and virus-induced exacerbations appear to peak during the winter months. This coincides with the increased prevalence of respiratory viral infection in the community and is also reflected in the increased utilisation of hospitals during winter (Donaldson and Wedzicha, 2006). In 2003 two studies reported a higher prevalence of respiratory

viruses in sputum and nasal lavage of COPD patients at exacerbation compared to in the stable state; 56% vs. 19%, $p < 0.01$ (Rohde et al., 2003) and 64% vs. 7% (Tan et al., 2003). This evidence together suggests that respiratory viral infection may be as, if not more, important than bacterial infection in triggering COPD exacerbations (Seemungal et al., 2001).

Earlier studies carried out in the 1970s and 1980s investigating the role of viruses in COPD exacerbations detected viruses in only 10% to 20% of exacerbations (Smith et al., 1980). The diagnostic tools used in these studies were less advanced than that of today and had low sensitivity, especially in the case of human rhinovirus (HRV). The development of modern molecular tests such as polymerase chain reaction (PCR) in 1983, has allowed further detection of viruses at more sensitive levels, allowing their association with COPD exacerbations to be more accurately defined (McManus et al., 2008). A study by Perotin and colleagues detected viruses in 44% of exacerbation samples using PCR techniques but in only 7% when using classical viral culture assays (Perotin et al., 2013). Viral infections are associated with more severe exacerbations in terms of a greater burden of symptoms which result in longer recovery times and greater likelihood of hospitalisation (Seemungal et al., 2000).

Although viruses have been detected in >50% of exacerbations, this number depends greatly on the technique used for detection. A study by Seemungal and colleagues in

2001 using nasal samples and quantitative PCR (qPCR) showed that 39% of all reported exacerbations were associated with viruses. Of these 66 detected viral exacerbations, 39 (59.0%) were associated with HRV, 17 (25.8%) with respiratory syncytial virus (RSV), 7 (4.2%) with coronavirus and 26 (3.0%) with influenza B (Seemungal et al., 2001). Of the 83 patients, 53 (64%) had at least one respiratory virus detected at exacerbation and these patients overall had higher exacerbation frequencies. Viral exacerbations were associated with increased dyspnoea and colds along with a median symptom recovery time of 13 days, longer than the typical 6 day recovery time seen in non-viral associated exacerbations (GOLD Report 2014). A separate study by Greenberg and colleagues, using serological and viral culture, showed that 23% of hospitalised COPD patients were infected with a virus and that the mean time to return to symptomatic baseline was 2 weeks (Greenberg et al., 2000). This result, which detects viral infection at a lower prevalence compared to other studies, strengthens the idea that viral culture does not detect the prevalence of viruses as efficiently or accurately as qPCR. It may suggest that the viral load in samples taken from exacerbating patients after admission to hospital may have decreased considerably by the time the sample is taken. Patients who are hospitalised will have often developed the viral infection a number of days before they are admitted to hospital so taking an “exacerbation presentation” sample after admission, may be an unfair representation of peak viral load as this may have occurred a number of days before the infection began.

1.2.4.2 Mechanism of viral infection in COPD

As mentioned previously (*section 1.1.2*), COPD is associated with proinflammatory cytokines and neutrophil chemokines such as TNF- α , IL-8, GRO- α and leukotriene B4 (LTB4). At exacerbation, inflammatory mediators are increased leading to exaggerated airway inflammation compared to patients in the stable state (Mallia and Johnston, 2006). Exacerbations are associated with the activation of NF- κ B in macrophages in sputum samples (Caramori et al., 2003) and it has been demonstrated, *in vitro*, that HRV infection can cause NF- κ B activation (Papi and Johnston, 1999). This is one potential mechanism in which respiratory viruses can up-regulate proinflammatory mediators in the airways leading to heightened inflammation. These mediators which have a chemotactic effect on neutrophils (IL-8, ENA-78 and LTB4), lymphocytes and monocytes (RANTES), and the up-regulation of adhesion molecules (TNF- α) may be key elements in the increased inflammation that occurs during a COPD exacerbation (Figure 1.5).

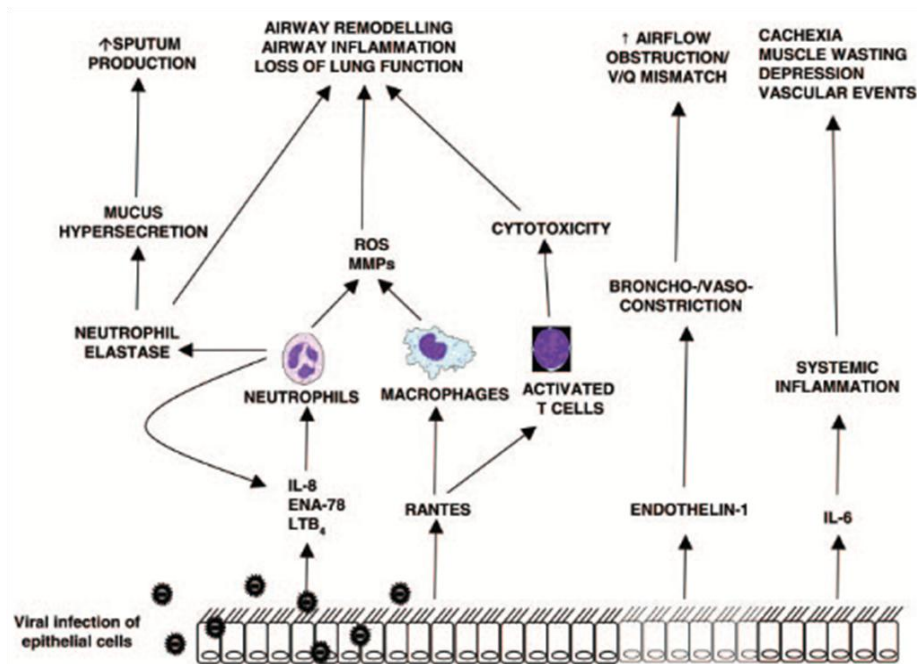


Figure 1.5: Mechanisms of virus-induced COPD exacerbations. Viral infection of epithelial cells leads to the release of proinflammatory cytokines and chemokines. Chemokines attract inflammatory cells that release toxic products, stimulating mucus production and leading to tissue damage with possible long-term loss of lung function. Some mediators such as endothelin-1 have a direct effect in causing bronchoconstriction and vasoconstriction, resulting in airflow obstruction and impaired gas exchange (Mallia and Johnston, 2006).

After an initial viral infection, cytokines are released by airway epithelial cells leading to an influx of inflammatory cells (Mallia and Johnston, 2006). The inflammatory cells release products such as neutrophil elastase and reactive oxygen species (as described in *section 1.1.3*). This in turn leads to tissue damage, increased mucus production and further cytokine release (Mallia and Johnston, 2006). Interestingly, some studies have reported increases in inflammatory cell counts at exacerbation compared to in the

stable state (Hurst et al., 2006; Mercer et al., 2005) where-as others have reported no differences (Bhowmik et al., 2000). A study by Hurst and colleagues in 2006 found a significant increase in total leukocyte sputum count at exacerbation ($6.47 \log_{10}$ cells/ml) compared to the stable state ($5.86 \log_{10}$ cells/ml) ($p < 0.001$) and also in nasal wash samples ($p = 0.043$) (Hurst et al., 2006). Other studies have reported increased neutrophil, eosinophils and lymphocyte counts from the stable state to exacerbation (all $p < 0.05$) (Mercer et al., 2005). One study by Papi and colleagues reported that levels of sputum neutrophils were increased during exacerbation compared to the stable state, regardless of aetiology, however the eosinophil count was only increased in patients found to have a viral infection (Papi et al., 2006). The variability and differences in the results found between these studies indicates the need for further study in carefully selected populations to determine exacerbation aetiology.

1.2.4.3 Human rhinovirus infection

There are a number of viruses that have been detected at exacerbation including coronavirus, RSV, influenza, parainfluenza and adenovirus, however, it has been shown that the most commonly detected virus is HRV (Seemungal et al., 2001a). HRV has been identified in up to 60% of virus-associated exacerbations (“GOLD Report 2014,” n.d.). Figure 1.6 adapted from work by Seemungal and colleagues illustrates HRV as the most prevalent virus at exacerbation highlighting the importance of it as an exacerbation trigger.

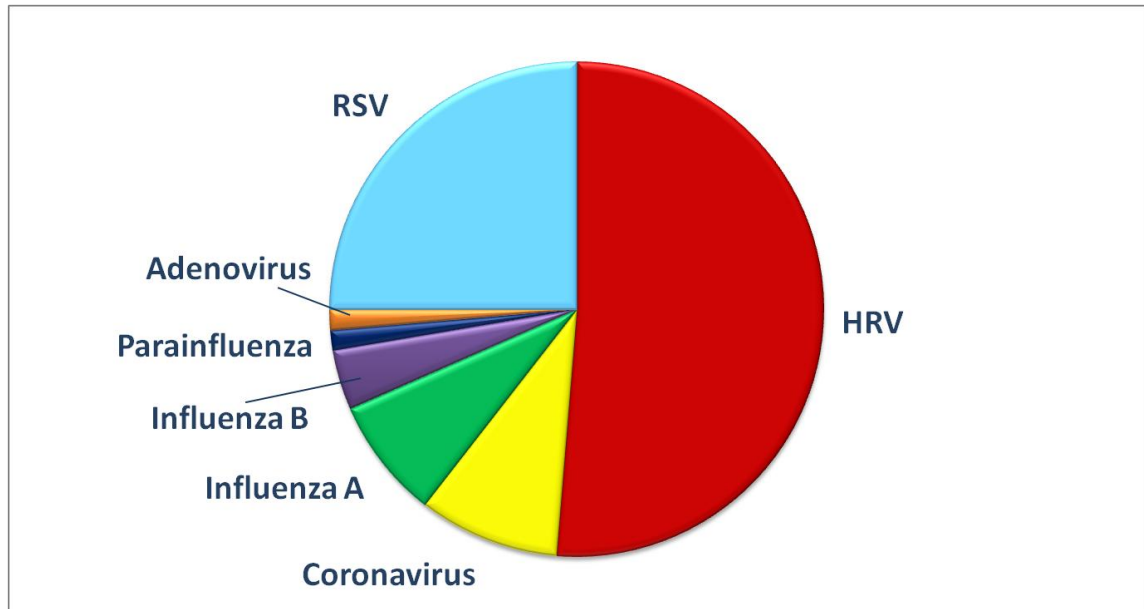


Figure 1.6: A breakdown of the respiratory viruses detected at COPD exacerbation (Seemungal et al., 2001)

HRV is one of the major groups of viruses that cause the common cold in the population worldwide. It belongs to the *Picornaviridae* family of which there are >100 serotypes (Flint et al., 2000). It is a small, non-enveloped icosahedral particle of 24-30 nm in size (Greenberg, 2003). The HRV virion contains a viral capsid which originates from a single polyprotein which is translated from the viral RNA genome and is proteolytically cleaved in the host cells. The capsid forms four virus-encoded proteins; VP1, VP2, VP3 and VP4. VP1 – 3 are found on the surface of the capsid whereas VP4 is found internally within the virus and is responsible for anchoring the viral RNA to the

centre of the capsid. Immunity to HRV is serotype-specific due to the variations in the VP1 – 3 surface proteins which leads to antigenic diversity and therefore limits the production of an effective vaccine (Flint et al., 2000). The capsid contains a single strand of positive sense RNA of approximately 7 kilobases in length which wraps around the viral genome. It is built from 60 subunits arranged as 12 pentamers. Each subunit contains the four capsid proteins in an identical arrangement. The capsids of HRV have deep canyons surrounding the 12 fivefold axes of symmetry in the pentamers and these canyons are the site of interaction with the cell surface receptor, ICAM-1 (Figure 1.7). The binding site on the capsid for the low-density lipoprotein (LDL) receptor is located on the star-shaped plateau at the fivefold axis of symmetry (Flint, 2004). The HRV virion attaches to the nasal or bronchial epithelium by one of these two epithelial receptors depending on whether the HRV serotype is part of the major or minor group. Over 90% of HRV serotypes belong to the major group and these serotypes bind with ICAM-1 on the epithelial cell surface whereas the minor group HRV serotypes bind with LDL receptors (Flint et al., 2000; Flint, 2004). Infection occurs when HRV successfully binds to the cell membrane via one of these two receptors. It then uncoats itself allowing the viral RNA to enter into the cell cytoplasm which is where the synthesis of virions occurs (Flint, 2004). Once in the cell, the viral RNA attaches to the host cell machinery and uses it to translate and synthesise the viral polyprotein and to replicate the viral RNA (Flint, 2004). Replication occurs using two, partially double-stranded RNAs called the replicative intermediates (RIs). Both the sense RNA strand

and antisense RNA strand are used as templates while the viral polyprotein is translated from the viral genome and undergoes multiple proteolytic cleavage to form both structural (VP1- VP4) and non-structural proteins. The four capsid proteins are then packaged with viral RNA to form the mature virion which is released out of the cell by cell lysis (Flint, 2004). The synthesis of RNA can be detected within 6-8 hours after the initial infection and within 48-72 hours, the production of viral RNA and viral particles, peaks (Flint, 2004).

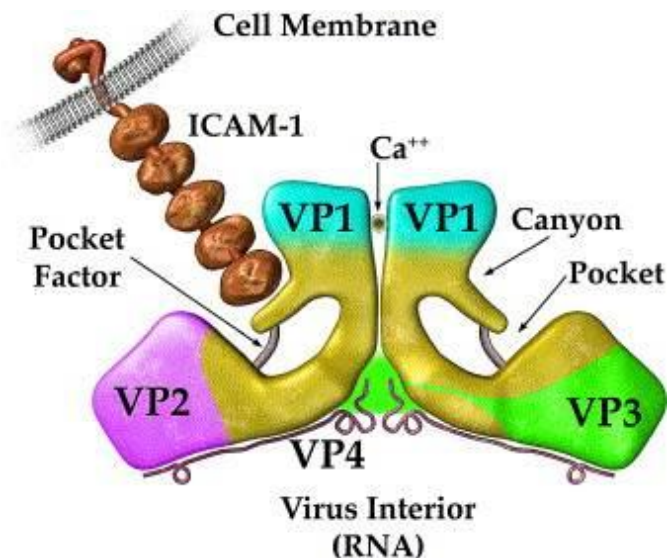


Figure 1.7: Transverse section through the centre of a pentamer depicting entry of its cellular receptor, ICAM-1 (Friedlander and Busse, 2005).

HRV is spread via direct contact and by aerosols (Monto, 2002) and can occur in all human populations, however it appears to cause more significant morbidity in those

who are immunocompromised, elderly or very young (Hicks et al., 2006; Monto et al., 1987). In the majority of the healthy population, HRV infection leads to a self-limiting illness that usually resolves by itself without any form of medical attention (Monto, 2002). Both upper and lower respiratory symptoms can be experienced with HRV infection including rhinitis, rhinorrhoea, sore throat, cough, sputum production and wheeze. Headaches, tiredness and fever are often present also (Monto et al., 2001). A number of studies have shown that HRV infection plays an important role in the respiratory tract of the elderly and contributes significantly to morbidity and mortality in this population (Hicks et al., 2006; Louie et al., 2005).

The respiratory conducting airways are divided into upper and lower airways at the levels of the larynx. Most HRV serotypes have an optimal growth temperature of 33°C which is the temperature of the nasal epithelium (Papadopoulos et al., 2000). A key issue regarding the role of viruses in the onset of exacerbations was whether upper respiratory viruses such as HRV could infect the lower airway as well as the upper. For many years it was thought that they could not due to the evidence suggesting the optimal temperature for HRV replication or growth was 33°C (Papadopoulos et al., 1999). However Papadopoulos and colleagues showed that HRV can replicate in the lower airway by using in situ hybridisation to exclude any sample contamination from the upper airway. They concluded that although most HRV serotypes replicate optimally at 33°C, the higher temperature of the lower airways is not a preventative

factor for their replication, and some strains may even prefer it (Papadopoulos et al., 1999). Other studies have shown positive detection of HRV using sputum samples suggesting that HRV can infect the lower airway as well as the upper. A study by Seemungal and colleagues in 2000 showed the prevalence of HRV to be 40% higher in induced sputum samples than in nasopharyngeal samples by PCR (Seemungal et al., 2000). Similarly, a study by Gern and colleagues in which subjects were experimentally infected with HRV found that before inoculation, none of the 23 lower airway samples were found to be positive for HRV, however after inoculation 80% of samples were positive (Gern et al., 1997). This evidence suggests lower airway infection by HRV is likely and therefore may contribute to virus-induced exacerbations of COPD.

Further data in support of HRV causing COPD exacerbations comes from a study by Mallia and colleagues in 2011 in which mild COPD patients were experimentally infected with HRV (Mallia et al., 2011). The study investigated the time-course of experimental HRV infection as a model of COPD exacerbations using 13 mild COPD subjects and 13 control subjects in which low-dose HRV was diluted and inoculated into both nostrils. The study measured changes in upper and lower respiratory symptoms, lung function, inflammatory markers in the blood and sputum, BAL and blood cell counts and HRV load in sputum and nasal lavage. It was found that upper respiratory tract symptom scores were significantly higher than baseline in both groups and that on days 13-16 post-inoculation scores were significantly higher in COPD patients

compared to controls. Between days 11 and 35 post-inoculation, lower respiratory tract symptoms were significantly greater in the COPD group compared with control subjects. IL-6 levels in BAL significantly increased from baseline in COPD subjects but not in controls and there was a significant increase in BAL lymphocyte percentage from baseline in COPD subjects but not controls. Sputum neutrophil levels increased significantly from baseline in COPD subjects but not controls and were found to be significantly higher in COPD patients compared to controls. IL-8 levels increased significantly from baseline in COPD subjects but no significant change was seen in control subjects.

HRV load was determined by qPCR techniques in induced sputum samples and in nasal lavage. HRV load in nasal lavage samples increased rapidly 48 hours after inoculation in both COPD and control subjects. However between Days 3-15, nasal lavage HRV load was higher in COPD subjects than controls and this was significant on Day 6. Sputum HRV load was higher in COPD patients compared to controls on Days 5 and 9 with the load peaking on Day 5. The HRV load remained significantly higher in the COPD subjects compared to controls, up until Day 15 (Figure 1.8).

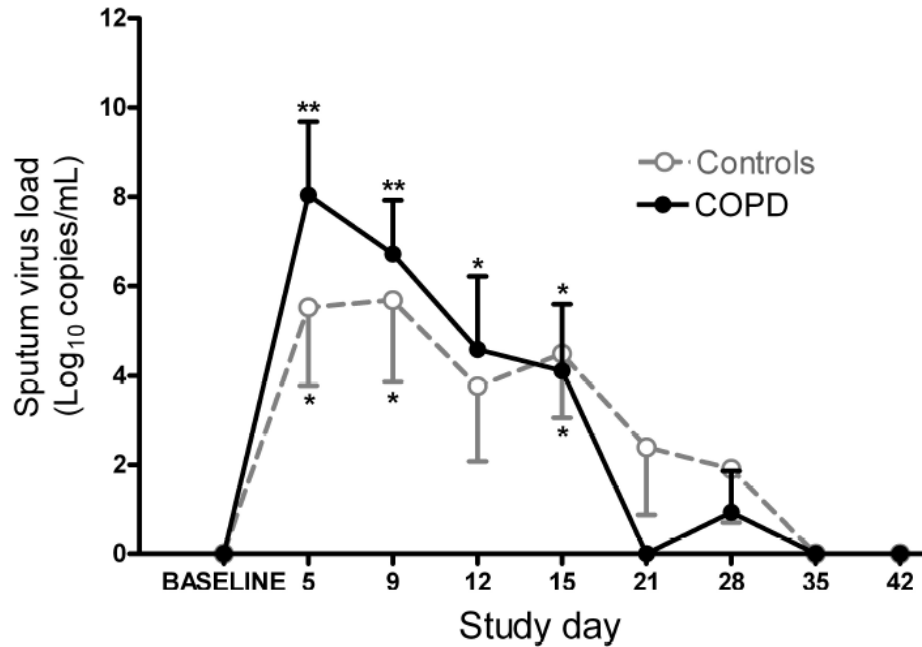


Figure 1.8: The time-course of experimental HRV load measured by qPCR at inoculation, exacerbation and during recovery in patients with COPD vs control subjects (Mallia et al., 2011).

This study shows that experimentally infecting COPD patients with HRV induces upper and lower respiratory tract symptoms, airflow obstruction, neutrophilic inflammation and an increase in inflammatory markers. This experimental work supports previous evidence for the role of HRV in triggering exacerbations (Mallia et al., 2011) however was limited to using mild COPD patients, infected with a low dose of virus.

Measuring the changes of HRV prevalence and load during the time-course of natural exacerbation and recovery has not yet been investigated, but is an important area to study to allow our knowledge of HRV infection in COPD to expand.

1.2.4.4 Respiratory Syncytial Virus

The work by Seemungal and colleagues in 2001 (Figure 1.6) showed HRV to be the most commonly detected virus at COPD exacerbation, however RSV was the second most commonly detected virus at exacerbation with 25.8% of viruses being RSV (Seemungal et al., 2001a). RSV contains a negative-sense, single-stranded RNA, and is an enveloped virus of the *Paramyxoviridae* family which has previously been recognised as a paediatric pathogen (Henderson, 1987). More recently however, RSV has become recognised as an important adult pathogen (Dowell et al., 1996) especially in older patients (Griffin et al., 2002), those who are immunocompromised (Englund et al., 1988) and in COPD patients (Falsey, 2005). In certain high-risk populations, RSV has been found to have a similar health burden as influenza and in the United States, around 10,000 deaths in over 65s are attributed to RSV infection each year (Thompson et al., 2003). As discussed in reference to HRV, the development of PCR techniques has allowed re-evaluation of the contribution of viruses such as RSV in diseases such as COPD. Previous methods of RSV detection using culture techniques were particularly difficult with RSV due to its thermolability (Falsey, 2007). ELISA techniques were found to be faster than culture however the low sensitivity and specificity of these methods

led to inaccurate results especially in subjects with low RSV loads (Falsey et al., 1996). Techniques such as PCR are only useful if they follow sound methods of sample collection; for RSV infection it seems that sputum is more sensitive than using nasal samples (Falsey, 2005).

Epithelial cells lining the airway are often the first cells to be targeted for viral infection and replication (Becker et al., 1992). Following an RSV infection via the respiratory epithelium, these cells produce increased levels of inflammatory and immunomodulatory molecules such as IFN- β and IL-1 α (Garofalo et al., 1996), IL-8 (Noah and Becker, 1993), IL-11 (Elias et al., 1994), IL-6 and TNF- α (Garofalo and Haeberle, 2000), and ICAM-1 (Stark et al., 1996) which are important in pulmonary defence. The production of these inflammatory and modulatory molecules is one in which epithelial cells contribute to cytokine generation, induction of the host cell antiviral state and leukocyte recruitment and activation. It may be due to the production of these molecules that RSV has been shown to be implicated as a cause of acute exacerbations of COPD (Ramaswamy et al., 2009). Figure 1.9 illustrates potential differences in the pulmonary defence against RSV in COPD patients compared to normal controls (Ramaswamy et al., 2009).

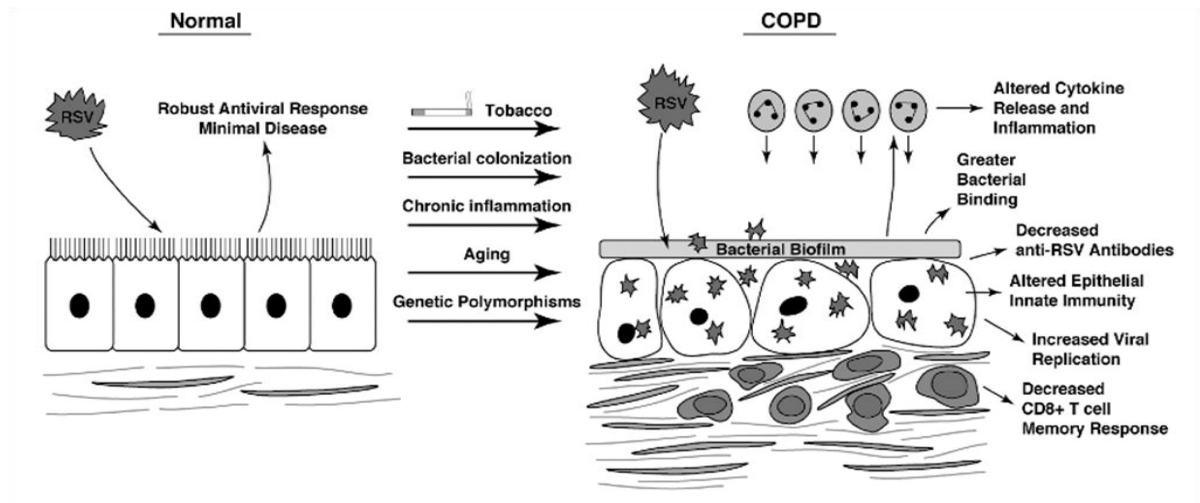


Figure 1.9: Potential differences in pulmonary defence against RSV in COPD patients. Factors that include age, environmental exposures, genetics, COPD itself (e.g. bacterial colonisation, chronic inflammation), or a combination of these may alter the host response to RSV thereby increasing susceptibility to or severity of infection. Altered defence responses include antiviral innate and adaptive, as well as inflammation that may lead to COPD exacerbation (Ramaswamy et al., 2009).

Like other enveloped viruses, RSV infection occurs by fusing its membrane directly with the endosomal or plasma membrane via cell surface receptors. This integration of cell surface receptors leads to activation of fusion at a neutral pH. In RSV, receptor binding and fusion are due to two major viral surface glycoproteins; G and F. The G protein is a highly glycosylated protein that is responsible for the binding of the virion to cells, whereas F, the fusion protein, includes membrane merging and the release of the viral capsid into the cytoplasm of the targeted cell through the fusion pore (Razinkov et al.,

2002). Once the viral nucleocapsid is released into the cell cytoplasm, RNA synthesis begins (Flint 2004).

Another mechanism that RSV can use to evade the host's immune system is to use the non-structural proteins (NS1 and NS2) to antagonise IFN- α , IFN- β and IFN- λ responses which results in impaired antiviral immunity and possible persistence of the virus (Spann et al., 2004). It also has the ability to escape an established immune response (Guerrero-Plata et al., 2006) as well as avoiding early abortion of infection via inhibition of apoptosis in the host cells (Krillov et al., 2000). These immune evasion and escape mechanisms could also help to explain persistence and subsequent lung damage contributing to COPD pathogenesis.

There are two distinct antigenic groups of RSV (RSV-A and RSV-B) however it is not clear whether infection by different strains affects COPD differently (Mufson et al., 1985). To date there is sparse data comparing the effects of RSV-A to RSV-B infection. However a study in which mice were infected with two different strains of RSV-A (Line A2 and Line 19) at similar viral titres, strain-specific variations in cytokine expression, goblet cell hyperplasia MUC5AC expression and airway hyper-reactivity were reported to be higher in the Line-19 strain compared to the A2. (Lukacs et al., 2006). In contrast, studies involving humans reported no differences in viral effects of infection with different RSV strains. In a study of elderly patients, RSV-A was responsible for 45%

of infection and RSV-B, 55% (Falsey, 2005). The main structural difference between the groups is in the G glycoprotein which is the protein that is proposed to be the most important in the induction of persistence (Tripp et al., 2001).

1.2.4.5 Viruses in stable COPD

Historically, the role of viruses in stable COPD has not been well studied. More recent studies however have reported that there is evidence for viral involvement in stable COPD as well as at exacerbation. RSV has been shown to be in approximately 23-28% of stable COPD subjects (Borg et al., 2003; Seemungal et al., 2001). One study reported that RSV viral RNA was persistent in 32.8% of 241 sputum samples from 74 subjects with moderate to severe stable COPD (Wilkinson et al., 2006). Studies comparing viral presence at COPD exacerbation and in the stable state have predominantly found that the prevalence is higher at exacerbation compared to in the stable state. However there have been studies that have shown a higher prevalence of RSV in the stable state (23.5%) compared to exacerbation (14.2%) (Seemungal et al., 2001). Persistent RSV in COPD is associated with higher levels of IL-6, IL-8, MPO and also faster lung function decline (Wilkinson et al., 2006). In contrast, other studies have not reported higher RSV prevalence in the stable state compared to exacerbation (Papi et al., 2006) and did not find RSV persistence in COPD (Falsey et al., 2006). This disparity could be due to a combination of factors including PCR technique, different PCR sensitivity cut off levels,

differences in the severity of COPD patients included in the study, and differences in the populations studied (Sikkel et al., 2008).

Adenovirus has also been reported to have a similar rate of detection in stable COPD and at exacerbation (McManus et al., 2007). Although it has been suggested in some studies that it is uncommon for adenovirus to cause latent or acute infection in COPD subjects (McManus et al., 2007), other studies have hypothesised that adenovirus may lay dormant in cells and may be involved in the pathogenesis of COPD. A study evaluating lung tissue from COPD patients found levels of adenovirus DNA to be higher than in matched healthy smokers (Matsuse et al., 1992). This latent adenovirus infection has been shown to increase the lung volume and airspace volume in a guinea pig model and decrease the surface to volume ratio when in combination with cigarette smoke compared to cigarette smoke exposure alone (Meshi et al., 2002). It has also been hypothesised that latent adenoviral infection could induce persistent inflammation in the lungs which results in emphysema. It has been shown that the adenovirus E1A gene is more highly expressed in both the alveolar and bronchiolar epithelial cells of COPD patients compared to controls (Hayashi, 2002). The presence of adenovirus and RSV in stable COPD may contribute to the pathogenesis of COPD as there are some common pathological features such as the large presence of CD8+ T lymphocytes that appear in respiratory viral infection as well as in COPD; however this hypothesis has not been proven to date. Studies of airway inflammation in stable COPD

patients have shown that the disease is characterised by infiltration of macrophages, neutrophils and CD8+ T lymphocytes and increased expression of cytokines, chemokines and adhesion molecules (Mallia and Johnston, 2006).

1.2.4.6 Bacteria

Historically, healthy lungs were thought to be a sterile environment and not associated with any form of microflora. The theory was that any inhaled bacteria were rapidly removed via phagocytosis by alveolar macrophages (Erb-Downward et al., 2011). However more recently it has been shown that the 'sterility' of the respiratory tract is not maintained in COPD patients. A study by Monso and colleagues reported that 25% of COPD patients in the stable state were colonised by bacteria, with this study using the protected specimen brush for microbiology sampling (Monsó et al., 1995). Further studies looking at sputum, brushings and bronchial lavage reported similar findings. Sethi and colleagues found that 34.6% of COPD patients had bacteria detected in BAL fluid (Sethi et al., 2006) and Banerjee and colleagues reported 40% of COPD subjects had bacteria in their sputum (Banerjee et al., 2004). The prevalence of bacteria has been shown to be significantly higher in stable patients with severe COPD (53.8%) compared to those with moderate (27.8%) or mild (11.55); $p=0.004$ (Zalacain et al., 1999). Bacterial species such as *Haemophilus influenzae* (*H influenzae*), *Moraxella catarrhalis* (*M catarrhalis*) and *Streptococcus pneumoniae* (*S pneumoniae*) (Lode et al., 2007) have been shown to be commonly associated with COPD particularly at

exacerbation, although they are able to be cultured in approximately one-third of stable COPD patients (Rosell et al., 2005). These three airway bacteria are referred to as “typical airway bacteria” as they are commonly associated with infection in COPD patients. The bacteria colonisation in the stable state of COPD is associated with the same organisms that are found at exacerbation. Bacteria have been found in clinically significant concentrations in the airways of 4% of healthy adults, 29% of patients with stable COPD, and 54% of patients at exacerbation (Rosell et al., 2005). The prevalence of typical bacteria has been reported to be significantly higher at exacerbation (56.5%) compared to in the stable state (44.2%); $p=0.024$ as has typical bacterial load, $10^{8.3}$ colony forming units/ml (cfu/ml) vs $10^{7.3}$ cfu/ml, $p<0.001$ (Figure 1.10) with *H influenzae* being the most prevalent species in both disease states (Garcha et al., 2012). This study also confirms that qPCR is significantly more discriminatory than culture at detecting these three main airway bacteria with 59.3% of samples being positive for bacteria using qPCR compared to 24.3% using culture; $p<0.001$ (Garcha et al., 2012).

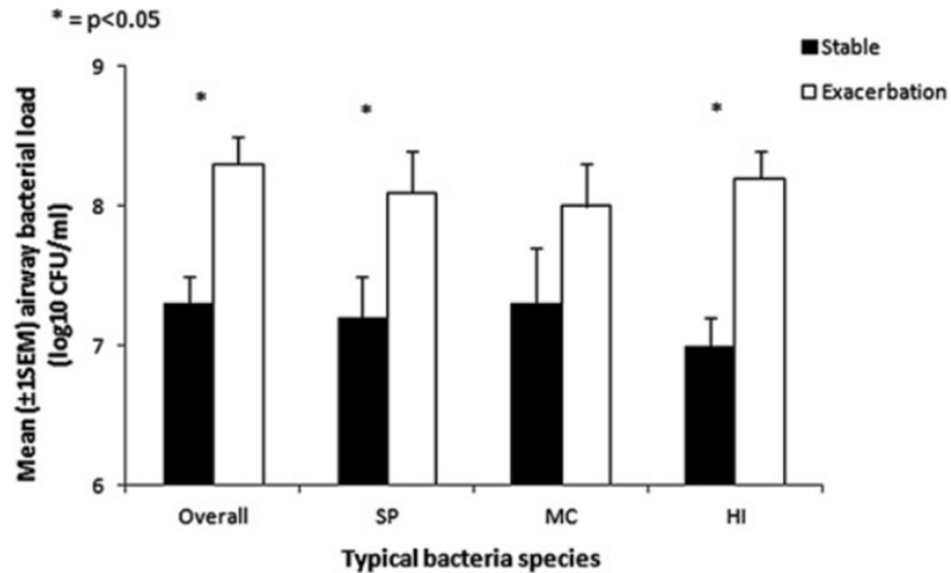


Figure 1.10: Quantitative PCR comparison of load of typical airway bacteria in stable COPD (n=242) and at exacerbation (n=131) in unpaired samples. Species: SP = *S pneumoniae*, MC = *M Catarrhalis*, HI = *H influenzae* (Garcha et al., 2012).

Although it is widely accepted that typical airway bacteria can trigger the onset of an exacerbation, the mechanism behind this is not so clearly defined. It has been hypothesised that it is the airway bacterial load that is the driver of exacerbations as it is strongly associated with inflammatory markers (Hill et al., 2000). Whether it is an increase in the load of already colonising bacteria that leads to an exacerbation or a new bacterial infection altogether is not clear. An alternative hypothesis suggests that intra-species switching of bacterial strains may be the cause of exacerbation onset; using molecular typing, isolation of a new strain of *H influenzae*, *M catarrhalis* or *S*

pneumoniae was shown to be significantly increased with a risk of an exacerbation ($p < 0.001$) (Sethi et al., 2002).

Bacteria have been shown to be associated with increases in inflammatory markers such as IL-6, IL-8, TNF- α and neutrophil elastase. The degree of inflammation was reported to be bacterial species-specific, with higher bacterial loads being associated with higher levels of lower airway inflammation (Hill et al., 2000). Bacteria themselves are able to produce products and proteolytic enzymes that cause local cellular necrosis and apoptosis which can lead to tissue damage (Abusriwil and Stockley, 2007) but are also able to activate different cell types in the lungs, triggering the release of chemokines and cytokines via various proinflammatory signalling pathways and the interferon pathway. Various cells including neutrophils, macrophages and lymphocytes are then recruited into the affected area which in turn can release neutrophil elastase and cathepsins (Abusriwil and Stockley, 2007). Some bacteria such as *Pseudomonas* and *Streptococcus spp.* are able to cause direct cellular effects with the induction of epithelial cell apoptosis and neutrophil necrosis which can cause further release of proteinases and proinflammatory mediators. This can lead to further impairment of host defences such as increases in mucus secretion and impaired ciliary function (Abusriwil and Stockley, 2007). A vicious cycle of infection and inflammation can be set up in which bacterial infection or colonisation leads to inflammation which results in further destruction of already impaired immune defences. This in turn leads to re-

infection or colonisation of the airway with bacteria (Sethi et al., 2009) meaning bacteria themselves can be a contributory factor in the persistence of inflammation in COPD (Abusriwil and Stockley, 2007).

Other bacteria, termed as atypical bacteria, such as *Chlamydophila pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* are infrequently identified in COPD patients using conventional culture techniques. A study using qPCR techniques to investigate the presence of atypical bacteria in stable and exacerbating COPD patients reported that out of 176 sputum samples (97 in the stable state, 79 at exacerbation) from 80 COPD patients, only 6 samples were positive (5 for *L pneumophila* and 1 for *M pneumoniae*) (Garcha et al., 2012). Similarly another study that used qPCR analysis to detect atypical bacteria colonisation in 248 sputum samples from COPD patients found just 1 sample (0.4%) to be positive for atypical bacteria (*L pneumophila*) (Diederer et al., 2007). This low level of detection suggests that the role of atypical pathogens in COPD is minor at both exacerbation and in the stable state and so do not require specific therapy.

1.2.5 Co-infection of virus and bacteria

Recently, the subject of viral and bacterial interactions at COPD exacerbation has become of considerable interest. Although COPD exacerbations are associated with

viral and bacterial infections, it is not known whether bacterial infection occur *de novo* or whether they are precipitated by viral infections. Few studies have reported on co-infection between bacteria and viruses at COPD exacerbation however all these studies support the hypothesis that one infection may follow the other (Cameron et al., 2006; Hutchinson et al., 2007; Kherad et al., 2010; Papi et al., 2006).

A study in 2006 performed by Papi and colleagues, showed viral and bacterial co-infection in 25% of samples (Papi et al., 2006) whereas Hutchinson and colleagues reported that 36% of exacerbations in which a virus was detected at onset, developed a secondary bacterial infection over the following 7 days, and overall, 78% of exacerbations in which *H influenzae* was isolated in the first 5 days after onset were preceded by viral symptoms (Hutchinson et al., 2007).

Patients hospitalised due to exacerbations have more marked lung function impairment and increased length of stay in the context of viral and bacterial co-infection than those with non-infectious exacerbations (Papi et al., 2006). Secondary bacterial infection may also play a part in exacerbation recurrence (Hurst et al., 2009) and further studies are required to assess the longer term effects of these key findings.

In 2006, Wilkinson and colleagues demonstrated that typical airway bacterial load was higher in the presence of HRV; exacerbations that were positive for both *H influenzae*

and HRV had increased bacterial loads ($10^{8.56(0.31)}$ cfu/ml) than exacerbations without both pathogens ($10^{8.05(0.77)}$ cfu/ml); $p=0.018$ (Wilkinson et al., 2006b). This may indicate that viral infections impact on the severity of exacerbations indirectly as they increase the bacterial load causing an extended or recurrent exacerbation in addition to the direct effects caused by the viral infection itself (Wilkinson et al., 2006).

A more recent experimental study exploring co-infection by Mallia and colleagues in 2012 examined whether HRV infection can precipitate secondary bacterial infection *in vivo*, and examined temporal relationships between the two using an HRV experimental model (Mallia et al., 2012). Moderate COPD patients and controls (healthy smokers and non-smokers) were experimentally infected with a low-dose of HRV. It was found that 60% of subjects with COPD developed a secondary bacterial infection, measured using culture techniques, after an initial HRV infection, measured using qPCR, which was significantly more frequently than in the controls. Sputum HRV load peaked on Days 5-9 post inoculation whereas bacterial load peaked on Day 15 (Figure 1.11). No patients were found to be positive for bacteria before the HRV infection which suggests that the bacterial infections were either due to bacteria that were present at levels below the sensitivity of culture in the stable state and that immune suppression due to the viral infection resulted in increased growth of these organisms to a detectable level, or that the bacterial infections were *de novo* infections due to acquisition of new organisms. Further studies need to be performed to

investigate the predominant mechanism behind the development of this secondary bacterial infection, including using alternative detection methods such as qPCR and sequencing for bacterial detection rather than culture, and including COPD subjects that have bacterial colonisation before an HRV infection.

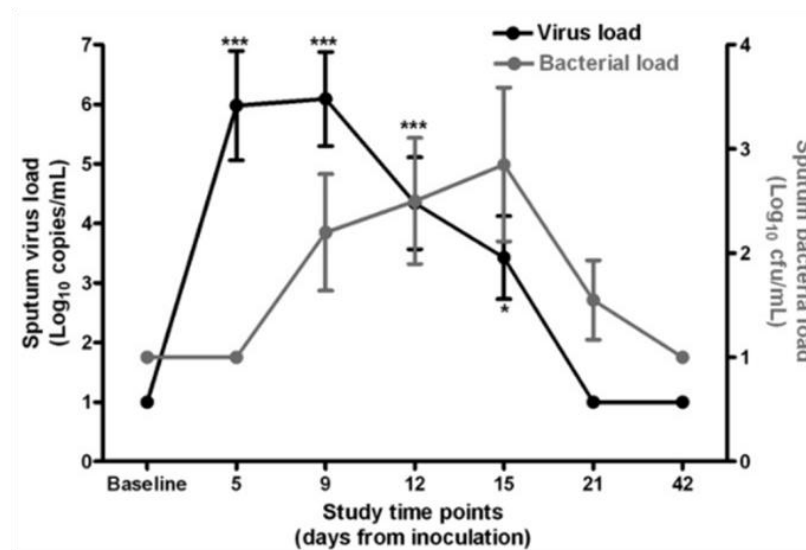


Figure 1.11: The time-course of sputum HRV load and bacterial load in COPD patients experimentally infected with HRV (Mallia et al., 2012).

The concept of viral and bacterial co-infection has also been explored when studying RSV infections. In 2006 Avadhanula and colleagues found that RSV (along with human parainfluenza virus and influenza) enhanced the adhesion of *H influenzae* and *S pneumoniae* to primary and immortalised cells lines. RSV infection increased the expression of several known receptors for pathogenic bacteria which in turn increased

the adherence of both these bacteria to airway epithelial cells under *in vitro* conditions (Avadhanula et al., 2006). It has also been shown that the G protein of RSV may work as a cell surface receptor for both *H influenzae* and *S pneumoniae* which therefore facilitates increased bacterial binding to virus-infected cells (Avadhanula et al., 2007). Together, this body of evidence suggests that HRV and to some extent, RSV, are able to interact with bacteria in ways that enhance severity during COPD exacerbation (Ramaswamy et al., 2009).

1.3 Aims and objectives

The hypothesis of this study is that HRV prevalence and load increase significantly from the stable state to COPD exacerbation. The overall aim of this thesis is to investigate the role of respiratory viral infection, in particular HRV, at COPD exacerbation and during recovery. This will be assessed by a number of different methods:

- ❖ Changes in the prevalence and load of HRV between the stable state and COPD exacerbation will be measured using a sensitive quantitative PCR (**Chapter 3**).
- ❖ The time-course of HRV prevalence and load during naturally-occurring COPD exacerbations and recovery will be explored (**Chapter 4**).
- ❖ The relationship between HRV load and upper respiratory tract symptoms in the stable state, at exacerbation and during recovery will be examined (**Chapter 5**).
- ❖ Investigation of the co-infection of HRV and typical airway bacteria during naturally-occurring COPD exacerbations and recovery (**Chapter 6**).
- ❖ Determine any associations between HRV infection and COPD patient reported outcomes such as EXACT and CAT scores (**Chapter 7**).

- ❖ Examine whether the presence of HRV during COPD exacerbation is related to exacerbation frequency (**Chapter 7**).
- ❖ Explore the changes in prevalence and load in semi-quantitative analysis of RSV in the stable state and at exacerbation (**Chapter 8**).

This will be the first study to investigate the role of HRV infection using viral load measurements during the complete time-course of naturally-occurring COPD exacerbations. By addressing these points I aim to provide significant contributions to our understanding of the role of HRV infection in naturally occurring COPD exacerbations.

CHAPTER 2. Materials and Methods

2.1 London COPD cohort

The London COPD cohort is a long-standing, rolling cohort of approximately 200 patients enrolled at any one time. The COPD patients are part of a long-term study in the Centre for Respiratory Medicine, Division of Medicine, at the Royal Free Hospital, University College London. The London COPD cohort is funded by the Medical Research Council, United Kingdom (Ref. G0800570).

2.1.1 Ethics and consent

Ethical approval for the London COPD cohort was granted by the Royal Free London National Health Service Foundation trust (Ref. 09/H0720/8), and patients gave informed written consent allowing sputum and blood samples to be collected, and spirometry readings to be taken. Approval was also given to allow daily diary card data to be obtained from patients.

2.2 Patient subjects

2.2.1 Recruitment

All 146 patients studied were recruited to the London COPD cohort, by cohort clinicians or by Beverly Kowlessar, the research nurse, if they had COPD defined by a number of criteria; all patients were either current or past smokers and were not recruited if suffering from other respiratory diseases. When seen by a London COPD cohort clinician, patients were informed about the cohort study and given the option to opt out and be removed from the study. They were also informed they were able to leave the study at any point. Upon recruitment, patients were given a unique study number to ensure confidential identification. Spirometry measurements were obtained using volumetric storage spirometer (Vitalograph 2160; Maids Moreton) to ensure they had COPD; a forced expiratory volume in one second (FEV_1) of $\leq 80\%$ of a normal value predicted from age, height and sex, and an FEV_1 : forced vital capacity (FVC) ratio of $\leq 70\%$ (Seemungal et al., 1998). On recruitment, the attending cohort clinician took a smoking history such as pack-year history and current smoking status along with a history of chronic symptoms such as dyspnoea, wheeze, cough and sputum production. Age, gender, height, weight, maintenance therapy details, daily respiratory symptoms, social history, number of times outside the house during a week, contact with children and the number of visitors the patient receives in their home were also recorded at recruitment. Occupational history

was recorded along with family history relating to COPD. Blood, urine and sputum samples were also collected from the patient. Patients were excluded if they were housebound and therefore unable to attend clinic appointments, or if they were unable to fill in daily diary cards. Patients with a history of any other significant respiratory diseases were also excluded. Patients had no treatment (either antibiotics or oral steroids) for the six weeks prior to recruitment.

2.2.2 Daily diary cards

At recruitment, patients were instructed on how to use daily diary cards provided by the clinic, to record a number of parameters, in order to monitor their condition. Each morning patients recorded their respiratory daily peak flow using a volumetric storage spirometer (Vitalograph 2160, Maids. Moreton, Buckingham, UK) which measures their maximal expiratory flow. The values recorded were compared to the reading taken whilst the patient was in the stable state, allowing any changes to be noted by the clinician at the next visit. In the evening, patients recorded how many hours they had spent outdoors each day, along with the number of footsteps they had taken using a pedometer (Yamax Digi-Walker SW-200). Any changes in symptoms experienced by the patient that day were recorded on the diary card as well as any changes in medication taken. An example of a daily diary card is shown in the appendix.

2.2.3 Clinic visits

Patients in the London COPD cohort were routinely seen in the research clinic every three months and their completed daily diary cards collected. These routine visits were defined as stable state visits providing there had been no exacerbation onset in the four weeks before, or during the two week interval after the visit. Once a year, patients underwent a comprehensive review when FEV₁ and FVC were measured with by spirometry and a history of smoking habits was taken.

Where possible, patients provided spontaneous sputum samples at each clinic visit during the stable state (3-month intervals), exacerbation presentation and recovery (3 days, 7 days, 14 days, 35 days and 56 days post-exacerbation). Figure 2.1 illustrates which patient samples were included in each analysis. Patients were given 24-hour phone access to a respiratory clinician and were taught to report any changes in symptoms as early as possible to ensure optimum conditions for sputum sample collection.

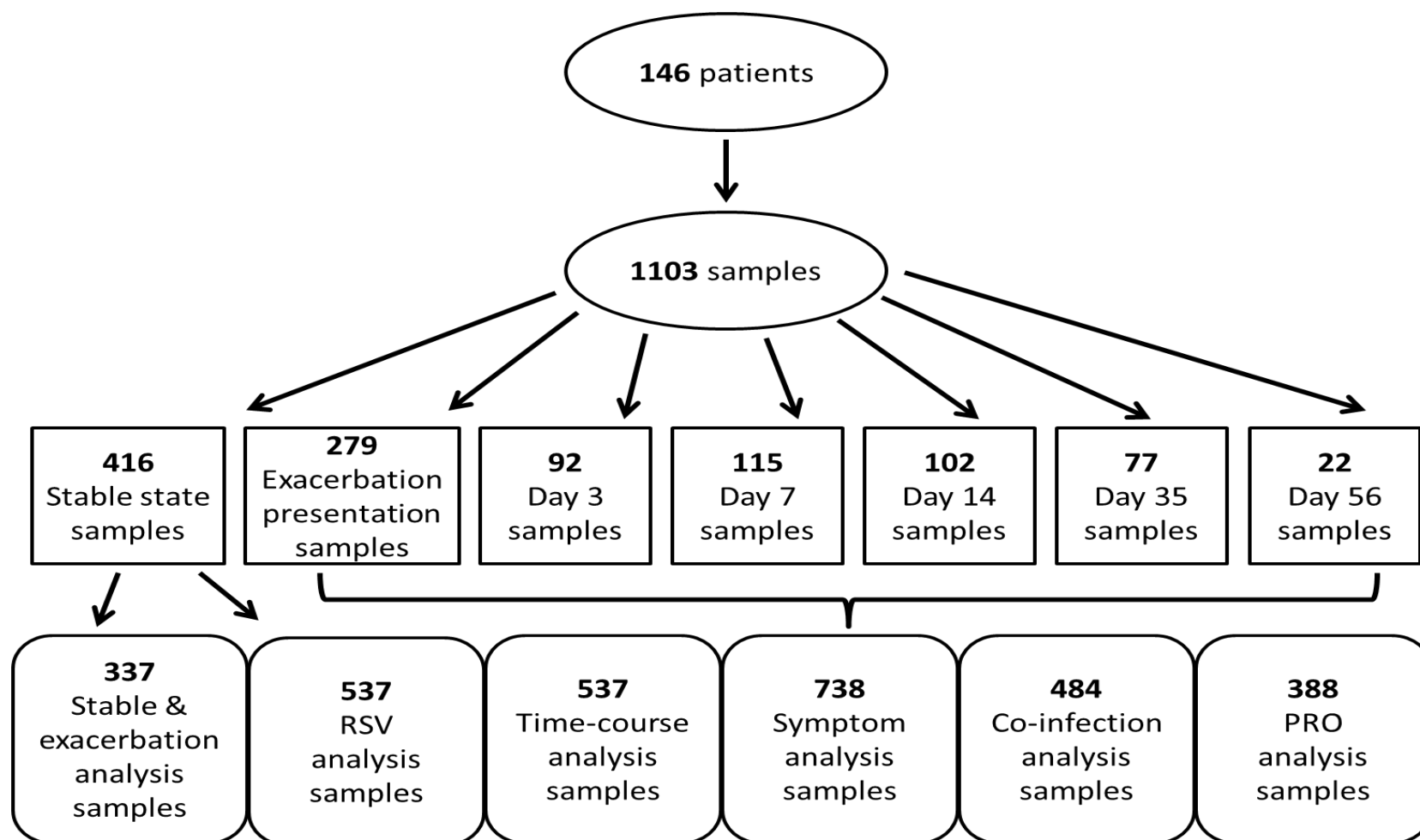


Figure 2.1: COPD patient groups analysed showing maximum number of samples available for each analysis. Patient samples may have been included in more than one sub-analysis

2.3 Definition of an exacerbation

Patients recorded increases in major or minor symptoms on their daily diary cards. Major symptoms included dyspnoea, increased sputum volume and increased sputum purulence and minor symptoms included wheezing, cold symptoms, cough and sore throat. This daily recording of symptoms was used to precisely determine the onset and recovery of exacerbations. Exacerbation onset was defined as the first of two or more days in which patients recorded a worsening of two or more symptoms with at least one being a major symptom. Symptoms were disregarded in identifying exacerbation onset if recorded continuously in the five day period preceding suspected exacerbation onset. Some exacerbations were identified in the absence of any diary card data; if the patient was admitted to hospital, had been treated by their GP or another physician outside of the study, or had used their rescue pack. A rescue pack is medication that is held by the patient at home which consists of oral antibiotics and systemic corticosteroids, usually for a week, to be started in case of the onset of an exacerbation. If an exacerbation was treated by any of these means, no sputum sample was taken from the patient.

Patients were strongly encouraged to contact the study team via the dedicated phone line should they experience any increase in their daily respiratory symptoms. They were attended to within 48 hours by a physician from the study team, their symptoms were

reviewed and the exacerbation was confirmed according to the symptomatic definition stated above. Expecterated sputum was collected in a sterile container from the COPD patients. The median time between the onset of the exacerbation and the sample being collected was 2 days. After the exacerbation sputum samples were taken from the patients, they were treated according to the prevailing guidelines (“GOLD Report 2014,” n.d.) and clinical judgment with increased inhaled therapy, antibiotics and/or oral steroids.

2.3.1 Exacerbation frequency

An annual exacerbation rate was calculated for each patient by dividing the number of exacerbations a patient experienced, by the number of years of diary card data in 2012 and 2013. This time period was chosen as it was contemporaneous with 90% of the samples. Patients with less than one year of diary card data were given an exacerbation rate equal to the number of events recalled in the previous year. Previous work has shown a good correlation between the number of exacerbations recorded on diary cards and the number of exacerbations recalled by the patient over the same 1 year period (Quint et al., 2010) and it has been shown that exacerbation frequency represents a relatively stable patient phenotype (Hurst et al., 2009).

2.4 Sputum

2.4.1 Collection

Patients expectorated sputum into a sterile pot after rinsing their oral cavity with water. If patients were unable to produce sputum spontaneously, they were induced by a cohort clinician or respiratory nurse. Before induction, oxygen saturations and pre-bronchodilator FEV₁ were measured and 200 mg Salbutamol was subsequently administered using a multidose inhaler (Ventolin, GSK). After 10 minutes, the post-bronchodilator FEV₁ was measured and patients were instructed to blow their nose and rinse their mouth out with water. In total, three sets of five minutes nebulisation (DeVilbiss UltraNeb2000 ultrasonic nebuliser) were performed with 3% saline (Pin et al., 1992). The aerosol output was approximately 2 mL/min with a mean particle size of 0.5-5 µm in diameter. After the initial five minutes of nebulisation, FEV₁ and oxygen saturation measurement was repeated for a further five minutes unless the FEV₁ had fallen by more than 20% compared with the post-bronchodilator value. After the second round of nebulisation if the FEV₁ had not fallen by more than 20%, the procedure was repeated for a third and final time. If at any point the fall in FEV₁ was greater than 20%, nebulisation was stopped immediately. Patients were able to expectorate sputum at any time during the induction process. The induced sputum

was collected in sterile sputum pots and processed in the same way as spontaneously expectorated sputum (Bhowmik et al., 1998).

2.4.2 Processing

The sputum samples produced were processed in the lab on the day they were obtained by either a cohort clinician or the laboratory manager (Raymond Sapsford). The sample was separated from contaminating saliva using disposable forceps and the weight of the sputum was recorded. The sputum was diluted eight-fold in phosphate buffer solution (PBS) (Sigma, P-4417). Approximately 15-20, 3 mm glass beads (33212 4G; VWR International Ltd, Poole UK) were added to the sputum-PBS before the mixture was homogenised by vortexing (Whirlimixer, Fisons, Ipswich, UK) for 15 seconds. The beads allow the cells within the sputum to be lysed so any intracellular viruses could be released and later detected. The homogenised sample was agitated on an IKA VIBRAX tube shaker (IKA Werke GmbH % Co. KG, Germany) for 15 minutes at room temperature (RTP) and then vortexed for another 15 seconds. The homogenised sputum was then aliquoted into 500 µl microcentrifuge tubes and stored at -80°C for subsequent analysis.

2.5 Real-time quantitative polymerase chain reaction

2.5.1 RNA extraction using TRI Reagent LS

RNA extraction involves the purification of RNA from biological samples, in this case sputum. The technique used in this study was the single-step total RNA isolation TRI Reagent LS (Sigma, T3809-LS) method (Chomczynski and Sacchi, 1987). It is a mixture of guanidine thiocyanate and phenol, and works alongside chloroform to homogenise or lyse biological samples separating them into 3 phases; an aqueous phase containing RNA, an interphase containing DNA and an organic phase containing proteins. Each component can then be isolated further. This is a very effective method of isolating intact RNA with little or no DNA or protein contamination. As the purification of RNA can be complicated due to the presence of ribonuclease enzymes that rapidly degrade the RNA in cells and tissues, all tubes and pipette tips used in the RNA extraction process and PCR set up were RNase/DNase free and were also placed under UV light for 30-40 minutes before use. All surfaces were cleaned with “RNaseZap” (Invitrogen, AM9782).

When required, samples were removed from the -80°C freezer and thawed at room temperature for 15-20 minutes. Once thawed, 750 µl of TRI reagent was added to the 500 µl of processed sputum. The samples were vortexed for 4 minutes and after 5

minutes incubation at RTP, 200 µl of chloroform (Sigma, C2432) was added and the samples vortexed for a further 4 minutes. The samples were incubated on ice for 10 minutes before being spun in a microfuge (Eppendorf, 5415C) at 12,000 x g for 15 minutes. The top aqueous layer containing the RNA was removed and transferred to a UV-sterilised 1.5 mL microcentrifuge tube and 500 µl of isopropanol (Sigma I9516) added. The samples were left on ice for 15 minutes before being centrifuged at 12,000 x g for 20 minutes. The supernatant was removed and 200 µl of 80% ethanol (Sigma E7023) added to the RNA pellet. After being spun in the microfuge for a further 5 minutes at 12,000 x g, the pellets were allowed to air dry for 30 minutes before being resuspended in 30 µl of RNase free water. Both positive (virus spiked sputum sample) and negative (water) controls were included in the RNA extraction.

2.5.2 Reverse Transcription

The extracted RNA was immediately reverse transcribed to generate complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814, Carlsbad, California). Prior to plate set up, the 2x reverse transcription (RT) master mix was prepared on ice. Loaded into each well of the 96-well PCR plate was 10 µl of RNA sample along with 10 µl of the 2x RT mastermix giving 20 µl reaction volumes (see table 2.1 for reagents included). All samples were run in duplicate along with controls: a negative water control, a positive control of highly

concentrated HRV1B and template control of the 2x RT mastermix with no RNA sample to ensure no contamination. The plate was sealed and loaded into the PCR machine (Techne TC-412 Thermal Cycler, Kieson). The RT programme was set up as follows; 10 minutes at 25°C, 2 hours at 37°C and 5 minutes at 85°C. Plates containing the converted cDNA were stored at 4°C until required for qPCR (maximum of 5 days storage).

Reagent	Volume (µl)
10x RT Buffer	2
25x dNTP mix (100 mM)	0.8
Multiscribe™ Reverse Transcriptase	1
10 x RT Primers	2
PCR Grade Water	4.2
RNA Sample	10

Table 2.1: Reagents used to make up 2x RT mastermix for reverse transcription.

2.5.3 Plaque assay – quantification of plaque forming units

As part of the current study, it was required that the HRV load present in a sample be recorded in plaque forming units per mL (pfu/mL) to more appropriately and easily relate it to previous literature and to ensure consistency of viral qPCR results within the department. In order to achieve absolute quantification of HRV load using qPCR, the generation of a standard curve was required. The standard curve was prepared using a plaque assay from tissue culture grown HRV1B (American Type Culture Collection, ATCC) using a HeLa Ohio cell line. At 75-80% confluency, the HeLa Ohio cells were passaged. The growth medium (GM) (Sigma, DMEM) was removed from the flask that contained the cells, and 20 mL of PBS added to the flask to “wash” the cells. Trypsin EDTA - 1% (Invitrogen) was then added to the flask in order to detach the cells enzymatically. After incubation for 3-4 minutes with 3 mL of trypsin, the cells detached from the flask surface. The trypsinisation was halted by the addition of 10 mL of GM before the cells were transferred from the flask to a 50 mL falcon tube and centrifuged at 250 x g for 7 minutes. The cells were then resuspended in GM and subcultured into more flasks at a dilution of 1:10 in order to continue their growth. For the plaque assay, the cells were seeded in 6-well plates (Sarstedt, Nümbrecht, Germany) at a concentration of 8×10^5 in 3 mL of maintenance media (MM) in preparation for the viral titration. The cell concentration was determined by adding 10 μ l of growth medium (GM) containing cells to 90 μ l of trypan blue (Sigma, T8154) which stains cells

blue allowing them to be counted using a haemocytometer (depth 0.1mm). Using x400 magnification, the cells were counted and the value multiplied by 10^5 to determine the number of cells/mL.

The cell concentration was determined using the following formulae:

$$\frac{8 \times 10^5 \times \text{number of wells needed}}{\text{number of cells/mL}} = \text{volume of cells required}$$

Total volume required – volume of cells required = volume of MM to be added.

The cells were incubated at 37°C and 5% CO₂ for 24 hours or until they reached confluency.

Serial dilutions of human rhinovirus (HRV) stock were prepared by dilution with PCR-grade H₂O in the range of 10^{-4} to 10^{-8} as shown in figure 2.2 below:

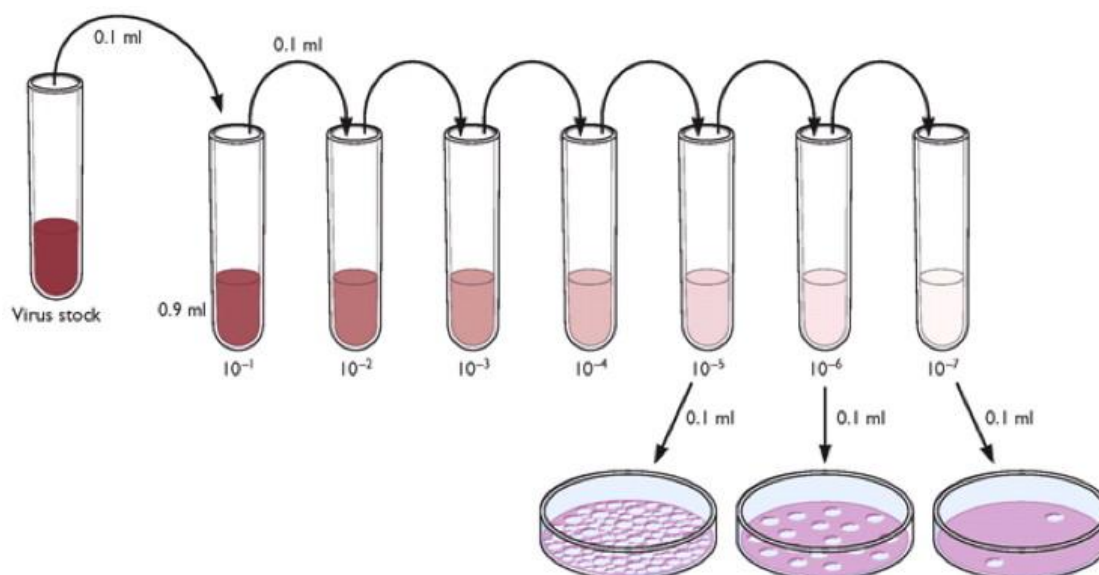


Figure 2.2. Serial dilutions of virus stock were made before adding to the cell monolayer in the 6-well plates. Taken from www.virology.ws.

The MM was aspirated from the wells without disturbing the cell layer and 200 μ l of the corresponding HRV dilution was added to each well. Each dilution was done in duplicate. A control (cc) was included where only media (without HRV) was added to the cells. The titration plate set up (Figure 2.3) is demonstrated below:

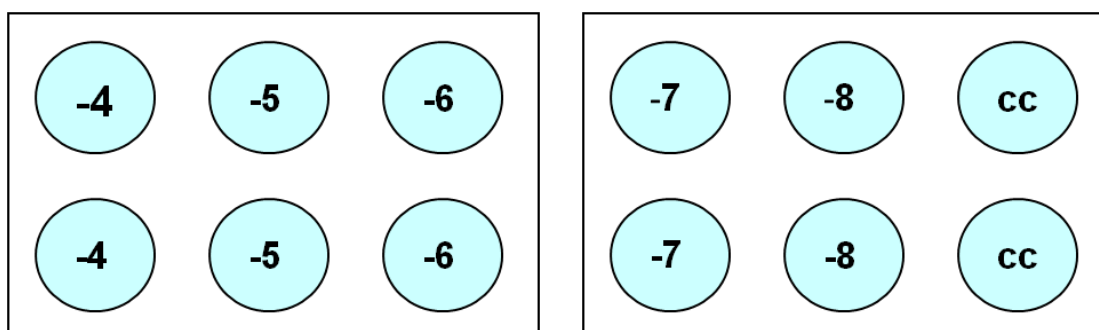


Figure 2.3: Titration plate set up to calculate HRV titre using the plaque assay method.

The plates were incubated for 2 hours at 33°C on a rocker allowing the HRV to infect the cells evenly. The HRV was aspirated from the cells and 3 mL of overlay medium (containing 800 µl of 1M MgCl₂) was added. The warm overlay medium containing 3% agar (Sigma, A5306) was allowed to cool and set. After 5 days of incubation at 33°C and 5% CO₂, 3 mL of 10% formalin was added to the cells and incubated at RTP for 1 hour. The agar plugs were removed without damaging the cell layer at the bottom. The cell layer was stained with 1 mL of 3,7-bis(Dimethylamino)phenazathionium chloride, Basic Blue 9, Tetramethylthionine chloride (methylene blue, M9140 Sigma) and incubated for 15 minutes at RTP. The stain was removed by aspiration and the plates inverted to allow the plaques to clear. The plaques appear as clear circles against a blue background as demonstrated in Figure 2.4.

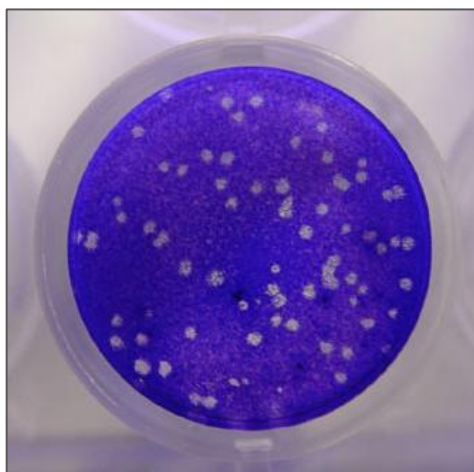


Figure 2.4: Example of a titration plate showing clear plaque in the cell monolayer. Taken from www.fr.wikipedia.org

The viral titre is a quantitative measurement of the biological activity of the recombinant virus and is expressed as pfu per mL. To calculate the viral titre, the number of plaques formed was counted at x200 magnification under the microscope.

The following formula was used to determine the titre (pfu/mL) of the viral stock:

$$\frac{\text{Number of plaques formed}}{d \times V} = \text{pfu/mL}$$

NB: d =dilution factor, V = volume of diluted virus added to the well

2.5.4 Standard curve preparation for HRV qPCR

As described above, the generation of a standard curve was required to achieve absolute quantification of the HRV load and was generated using HRV stock at a concentration of 1.35×10^7 pfu/ mL (calculated by plaque assay, *section 2.5.3*). The HRV stock underwent serial dilutions from 10^7 to 10^{-2} and each dilution was assessed by qPCR in triplicate. The cycle threshold (Ct) value (the number of PCR cycles required to reach a threshold fluorescence) of each sample was plotted against the concentration of each sample generating a standard curve (Figure 2.5).

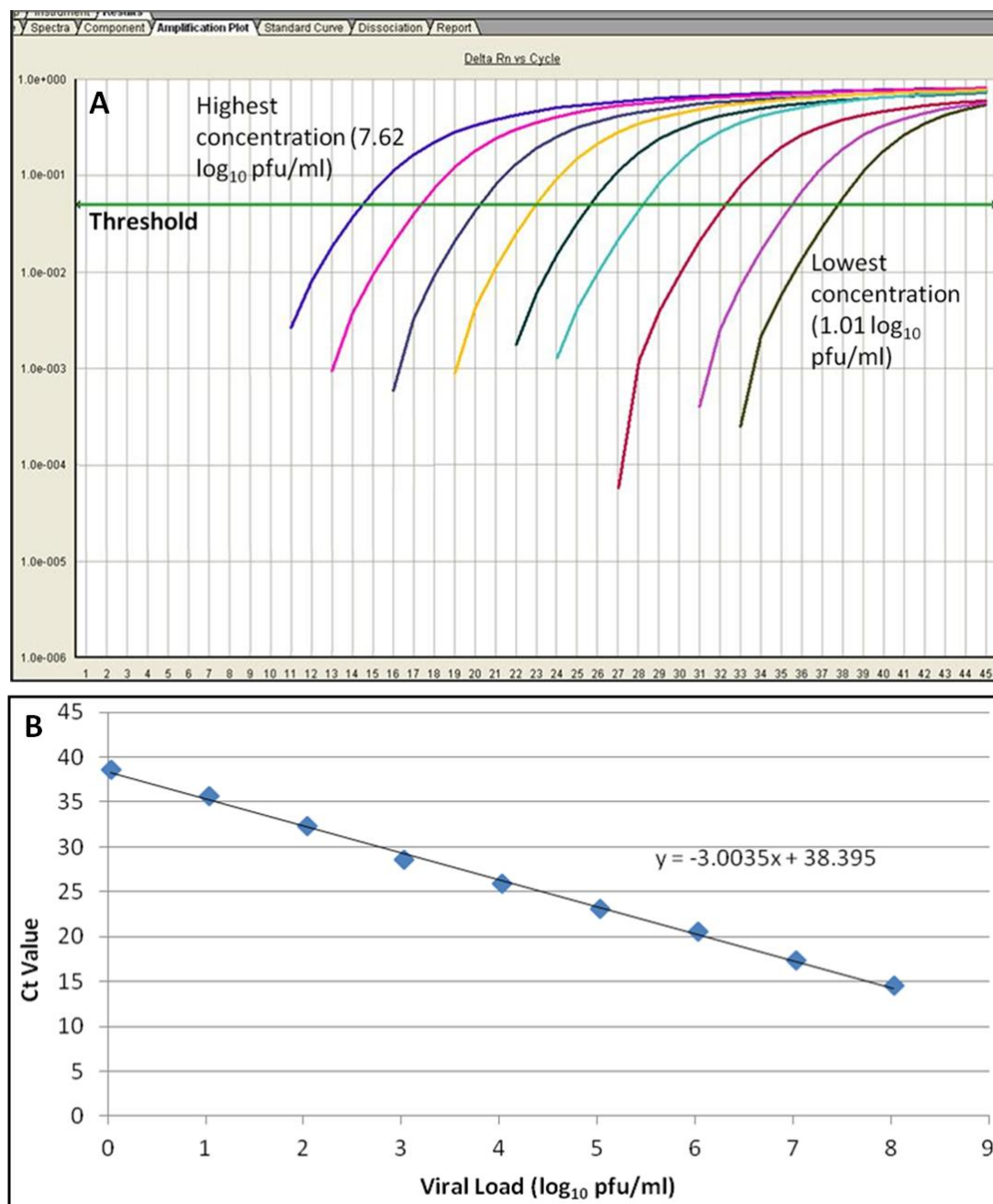


Figure 2.5: Standard curve generation for absolute quantification of HRV load. (A) Serial dilutions of known quantities of cDNA ran on PCR. The concentration ranged from $7.62 \log$ pfu/mL, down to $1.01 \log$ pfu/mL **(B)** Standard curve generated, relating cDNA concentration to Ct value. The equation for the linear regression line (shown on the graph) allows the quantity of an unknown sample to be determined.

2.5.5 Quantitative HRV PCR

Real-time qPCR assays were developed and optimised to allow rapid and sensitive detection of viral genetic material in clinical samples. Thermocycling and real-time detection of qPCR products were performed using the ABI Prism 7500 real-time qPCR System (Applied Biosystems). An internal amplification control (IAC) was incorporated in the qPCR to detect any PCR inhibition. The IAC selected here targeted the *phyB* gene from the potato species *Solanum tuberosum* (known as SPUD) (Nolan et al., 2006). It was incorporated in the PCR mastermix in order to detect any inhibition occurring in individual samples. This gene was previously validated as an IAC (Nolan et al., 2006). Details of SPUD primers and probe can be found in Table 2.6. The mastermix illustrated in Table 2.5 formed the basis of each PCR reaction. Into each well of the 96-well PCR plate, 22.5 µl of mastermix was added to 2.5 µl of cDNA sample creating a total reaction volume of 25 µl. A standard curve was previously created as described in section 2.5.4. Each PCR run included two standards as a positive control ensuring each PCR plate was run successfully. The standards also allowed the absolute quantification of the HRV load in any positive samples. The controls also included a negative control (no cDNA sample) and a control containing no QuantiTect Probe PCR Master Mix. All HRV samples were run in duplicate and the lower limit of detection for the HRV qPCR was 10.23 pfu/ mL ($1.01 \log_{10}$ pfu/ mL). This was achieved by examining the standard curve and identifying the lowest concentration at which the cycle threshold is crossed.

The qPCR conditions for HRV were set up as follows; heating to 95°C for 15 minutes activating the Taq polymerase, 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension for 80 seconds at 58°C Table 2.2). The primers and probes for HRV RT-PCR were based on protocols developed previously in the department and bind within the untranslated region of the genome sequence. The primers used were generic primers that were designed to detect as many HRV serotypes as possible including both major and minor types (Jacobs et al., 2013). The primers and probe sequences are shown in Table 2.6. Standard curves were developed by running PCRs with serial dilutions of known concentrations of virus (*section 2.5.4*).

Temperature (°C)	Time	
95	15 minutes	
95	15 seconds	} x45 cycles
58	80 seconds	

Table 2.2: The qPCR conditions for HRV primers and probe.

2.5.6 Semi-quantification of RSV

As the RSV qPCR was required to be semi-quantitative, there was no need to generate a standard curve. Relative quantification was used for RSV rather than absolute quantification as was used for HRV, where Ct values were compared to determine relative viral loads between samples. The upper and lower limits of RSV detection by qPCR needed to be measured. For the RSV-A, serial dilutions of neat RSV A2 stock (1×10^7 pfu/ mL) donated from Dr Maximillian Habibi at Imperial College London were set up from 10^7 to 10^2 (Figure 2.6A). For the RSV-B, serial dilutions of neat RSV-B stock, obtained from ATCC, were set up from 10^7 to 10^2 (Figure 2.6B). These dilutions were used to spike sputum samples which underwent RNA extraction, reverse transcription and qPCR to determine the upper and lower limits of RSV-A and -B detection.

The qPCR conditions for the semi-quantification of RSV-A were set up as follows; heating to 95°C for 15 minutes activating the Taq polymerase, 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension for 60 seconds at 57°C (Table 2.3). For RSV-B the set up was as follows; heating to 95°C for 15 minutes activating the Taq polymerase, 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension for 60 seconds at 60°C (Table 2.4). The primers and probes for RSV RT-PCR were developed by Bill Carmen's lab in West of Scotland virology service (Perkins et al., 2005) and are shown in Table 2.6. The RSV genome (ID: U39661) was

used as a source for primer3 software (www.primer3.ut.ee) to locate where the RSV primers bind to. The forward and reverse primers for RSV bind within the pneumovirus nucleocapsid protein gene (ID: AAC57032).

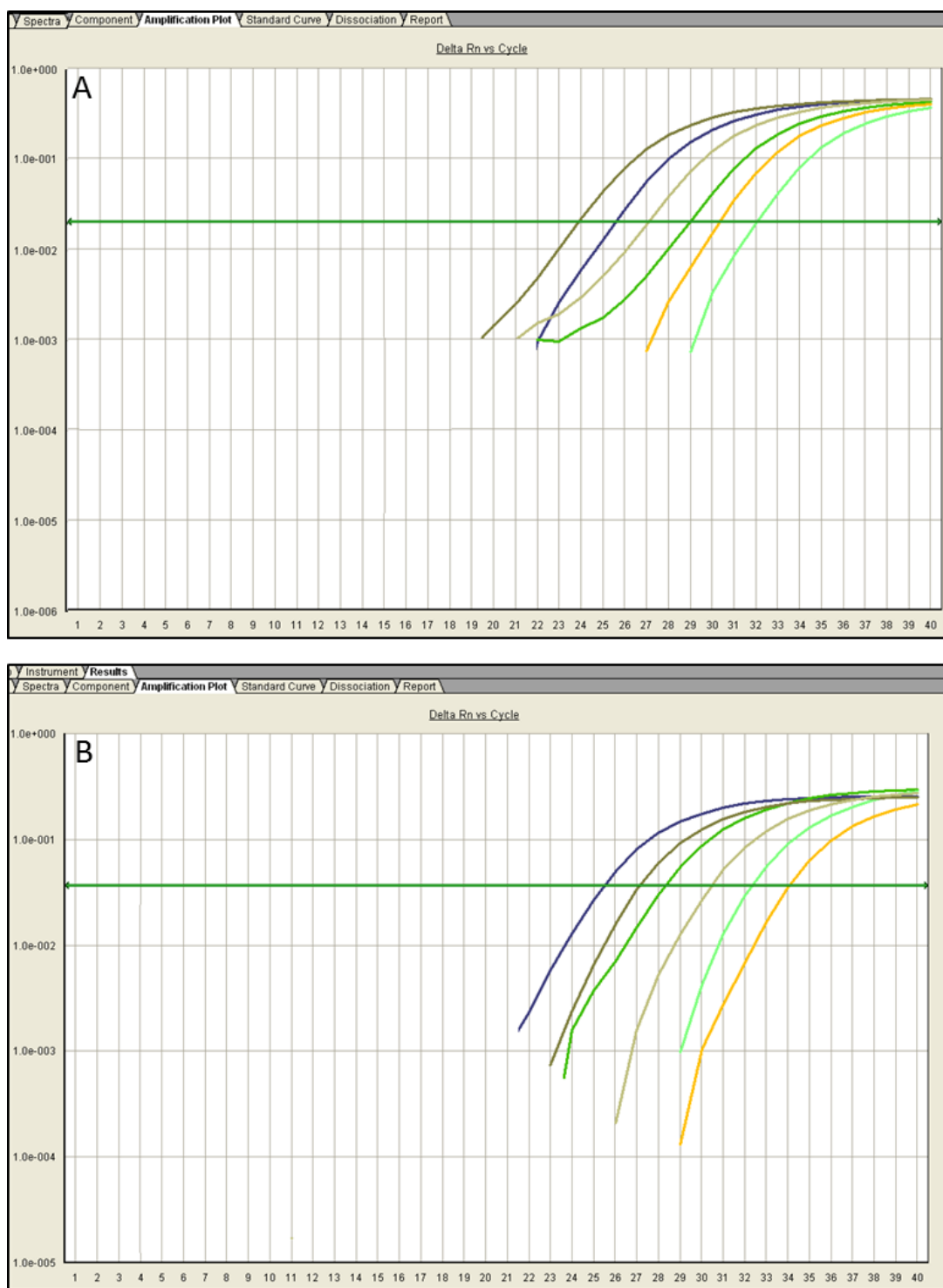


Figure 2.6. Dilution curve generation for semi-quantification of (A) RSV A and (B) RSV B. Serial dilutions of cDNA were run on PCR.

Temperature (°C)	Time
95	15 minutes
95	15 seconds
57	60 seconds

x40 cycles

Table 2.3: The qPCR conditions for RSV-A primers and probe.

Temperature (°C)	Time
95	15 minutes
95	15 seconds
60	60 seconds

x40 cycles

Table 2.4: The qPCR conditions for RSV-B primers and probe.

Reagent	Volume (μl)
QuantiTect Probe PCR Master Mix	12.5
HRV/RSV Forward Primer (20 μl)	1
HRV/RSV Reverse Primer (20 μl)	1
HRV/RSV Probe (20 μl)	0.35
PCR Grade Water	6.35
cDNA Template	2.5
IAC	1
IAC Forward Primer (50 μl)	0.125
IAC Reverse Primer (50 μl)	0.075
IAC Probe (50 μl)	0.1

Table 2.5: Reagents used to make up the mastermix for qPCR. IAC = internal amplification control.

Oligo Name	Sequence (written 5' - 3')
<u>Primers</u>	
HRV – Forward	TGADTCCTCCGGCCCCCT
HRV – Reverse	AAAGTAGTYGGTCCCRCTCC
RSVA – Forward	AGATCAACTTCTGTCATCCAGCAA
RSVA – Reverse	TTCTGCACATCATAATTAGGAGTATCAATT
RSVB – Forward	AAGATGCAAATCATAAATTCACAGGA
RSVB – Reverse	TGATATCCAGCATCTTTAAGTATCTTTATAGTG
IAC – Forward	AACTTGGCTTTAATGGACCTCCA
IAC – Reverse	ACATTCATCCTTACATGGCACCA
<u>Probes</u>	
HRV – Probe	6-FAM – AATGYGGCTAACCT – MGB
RSVA – Probe	FAM – CACCATCCAACGGAGCACAGGAGAT – BHQ-1
RSVB – Probe	FAM – TTCCCTTCCTAACCTGGACATAGCATATAACATACCT – BHQ-1
SPUD – Probe	Cy5 – TGCACAAGCTATGGAACACCACGT – BBQ

Table 2.6: Primer and probe sequences for quantitative HRV and RSV RT-PCR (purchased from Applied Biosystems UK), including IAC primers and probe sequences (purchased from TIB MOLBIOL).

Bacterial qPCR methods were carried out by Dr Davinder Garcha (Garcha et al., 2012).

2.5.7 HRV qPCR optimisation

To ensure consistency between results, primers and probe that had already been developed and used within the department were to be used for HRV detection in this study. The protocol was optimised by performing RNA extraction, reverse transcription and qPCR on sputum samples spiked with pure HRV16 at different concentrations, on water samples spiked with HRV16 at different concentrations and on water samples without any virus. This did not seem to yield positive results on the PCR which could have been due to a number of factors; 1) primer/probe set had been degraded, 2) viral stock used for spiking was no longer viable or 3) RNA contamination/degradation. These factors were resolved on a step-by-step basis. Dr Nathan Bartlett from Imperial College provided some cDNA to allow the already obtained primers and probe, to be tested. As this produced successful results, it was clear the problems lay within the RNA extraction or reverse transcription steps. The water sample that wasn't spiked with virus was showing positive results on the PCR suggesting contamination during one or more parts of the protocol. The aim was to go through each reagent used until the source of the contamination was eliminated. The first reagent used during the RNA extraction is TRI reagent and it was soon realised that it was the TRI reagent that had become contaminated with HRV and was therefore contaminating all the samples, and making them show up as false-positive for HRV on the PCR. However the PCR showed similar results in terms of HRV load for samples spiked with neat HRV and those spiked

with lower concentrations. This suggested that the HRV being detected was from the contamination from the TRI reagent as opposed to from the HRV16 spike. Dr Nathan Bartlett of Imperial College London kindly donated some HRV1B which was used to spike sputum and water samples. The protocol was run again and this time the PCR showed differences in the HRV load in samples spiked with different amount of virus suggesting the HRV16 was non-viable. Fresh HRV stocks were ordered from ATCC which were used in setting up the standards for the qPCR.

To illustrate the repeatability of the qPCR, the Ct values from the two duplicate PCR runs (run 1 and run 2) for 30 HRV-positive samples were plotted against each other (Figure 2.7).

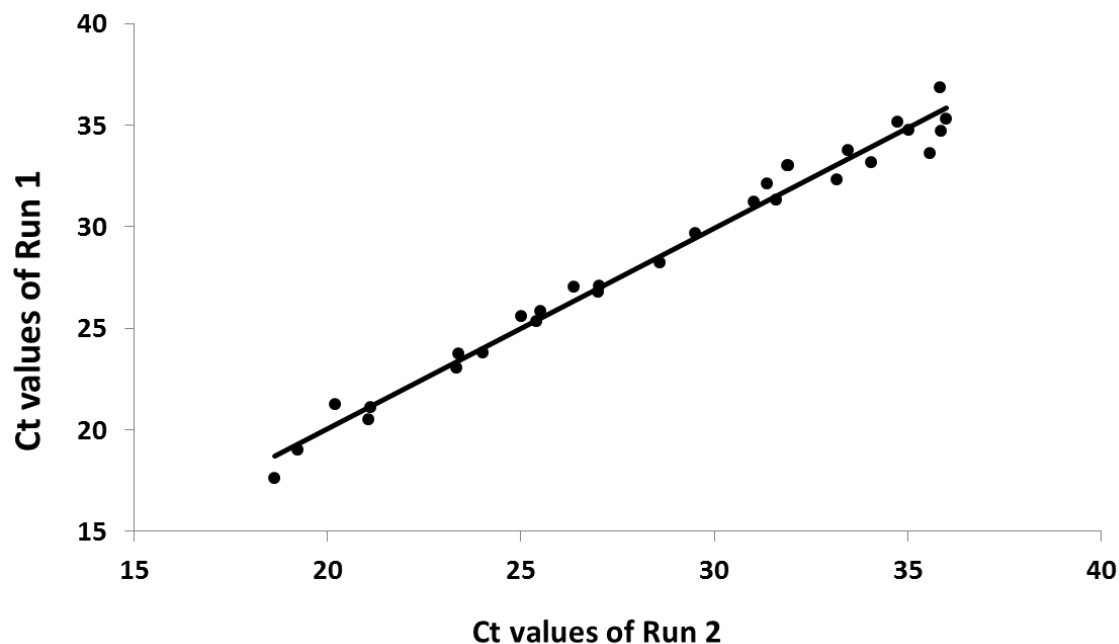


Figure 2.7: Repeatability curve showing Ct values for 30 HRV-positive. Each sample was run in duplicate to obtain a Ct value “run 1” and “run 2”.

2.6 EXACT Questionnaire

Permission to use the EXACT questionnaire to score symptom intensity was obtained from United BioSource Company (UBC, Bethesda, MD, USA). For use in this study, patients completed a paper version of the EXACT questionnaire at each clinic visit, based on the symptoms they experienced on the day of completion. An example of the EXACT questionnaire is shown in the appendix.

2.7 CAT Questionnaire

Patients completed a paper version of the CAT questionnaire at each clinic visit, based on the symptoms they experienced on the day of completion. An example of the CAT questionnaire is shown in the appendix.

2.8 Aushon Multiplex Immunoassay

Levels of biomarkers in sputum samples were measured using the Aushon multiplex immunoassay platform (Aushon BioSystems, Billerica, MA). Samples were incubated for one hour on the array plates that were pre-spotted with capture antibodies specific for each protein biomarker. Plates were decanted and washed four times before adding a cocktail of biotinylated detection antibodies to each well. After incubating with detection antibodies for 30 minutes, plates were washed four times and incubated for 30 minutes with streptavidin-horseradish peroxidase conjugate. All incubations were done at room temperature with shaking at 200 RPM. Plates were again washed before adding a chemiluminescent substrate. The plates were immediately imaged using the Aushon Cirascan CCD Imaging System, and data was analysed using Aushon Cirasoft Software in collaboration with Novartis.

2.9 Statistical analysis

Data were analysed using PASW Statistics V.21 SPSS (IBM Corporation, New York, USA). The Kolmogorov-Smirnov test for normality was applied; a non-parametric test that assesses the equality of continuous, one-dimensional probability distributions. HRV loads were reported as median and interquartile range (IQR). For graphical illustrations, \log_{10} transformed data were presented as mean ($\pm 95\%$ CI) or median (IQR). Non-parametric data were analysed using non-parametric statistical tests such as Mann-Whitney U tests and Wilcoxon tests for unpaired and paired data, respectively. Parametric data were compared using t-tests. Correlations between continuous variables were analysed using Spearman's Rank or Pearson's correlation in a univariate analysis. A probability value of $p < 0.05$ was considered to be statistically significant.

CHAPTER 3. HRV prevalence and load in stable COPD and at exacerbation

3.1 Introduction

This study aimed to assess changes in HRV prevalence and load from patients in the London COPD cohort, in the stable state and at COPD exacerbation, using real-time qPCR in naturally occurring exacerbations.

Patients in the London COPD cohort are seen in clinic every 3-6 months whilst in the stable state. Some patients in the COPD population are unable to spontaneously expectorate sputum in the stable state whereas others are able to produce it spontaneously. It is often the patients that suffer from chronic bronchitis which are able to produce sputum, as chronic bronchitis is associated with chronic spontaneous sputum production for at least 3 months of the year, for 2 consecutive years (GOLD Report 2014). However, patients that have a more emphysematous phenotype are often unable to spontaneously produce sputum. At exacerbation however, approximately 50% of patients in the London COPD cohort are able to spontaneously expectorate sputum (Patel et al., 2012). As discussed previously, the major symptoms defining the onset of an exacerbation are increased sputum volume, increased sputum purulence and increased dyspnoea.

Compared to the number of studies reporting viral infection at COPD exacerbation, there are fewer studies that focus on the presence of viruses in stable COPD, however viruses have been shown to be detected in the stable state. Seemungal and colleagues reported

that 16.2% of stable COPD samples had a respiratory virus present using quantitative PCR methods, and that in 45% of the virus-positive samples, human rhinovirus (HRV) was detected. Furthermore, it was shown that patients in which a respiratory virus was detected in the stable state, had a significantly higher exacerbation frequency than those without virus detected ($p=0.043$) (Seemungal et al., 2001).

More commonly, viral infection, in particular HRV, has been shown to be associated with exacerbations in both asthma (Friedlander and Busse, 2005) and COPD (Mallia et al., 2011; Quint et al., 2010; Seemungal et al., 2001; Stock, 2014; Wu et al., 2014). Viral detection in sputum samples, has been shown to increase at exacerbation compared to the stable state, 56% vs 19% ($p<0.01$) (Rohde et al., 2003). Liao and colleagues also found an increase in HRV prevalence from the stable state to exacerbation in sputum samples (5.3% vs 21.9%) but found a lower prevalence in both disease states when using nasal samples (3.5% vs 13.2%) (Liao et al., 2014). Furthermore, in a study by Tan and colleagues, viral detection in nasal lavage samples using PCR, was shown to increase from 7% in the stable state to 64% at COPD exacerbation (Tan et al., 2003).

In a study using culture techniques as opposed to PCR for virus detection, only 10% to 20% of exacerbations were found to be positive for the presence of a virus (Smith et al., 1980). Additionally, Beckham and colleagues showed that 57% of 88 respiratory viral infections were identified using culture or serological techniques with RT-PCR techniques identifying

a further 38 infections (Beckham et al., 2005). Although these differences in viral prevalence could be due to factors such as differences in sampling times where HRV has been shown to be more prevalent in the winter months compared to the summer (Wedzicha and Seemungal, 2007), or that samples taken from different geographic populations may have different HRV detection rates, the differences in viral detection rates between these studies suggest that culture techniques are less sensitive at detecting viral presence compared to PCR and that PCR is a more accurate method of determining the presence of viruses. Consequently, in the current study, the prevalence and load of HRV in both the stable state and at exacerbation were measured using qPCR techniques. A further potential limitation of previous studies include the method of data analysis with some studies using only unpaired analysis between stable and exacerbation states resulting in a potential population bias (Monsó et al., 1995; Rosell et al., 2005). In this study, the data were analysed using unpaired analysis in the overall population as well as paired analysis in a smaller subset of patients that were able to produce sputum spontaneously in both disease states.

In order to further the current knowledge of HRV infection in COPD and potentially reduce the frequency of exacerbations, this study aimed to assess changes in the prevalence and load of HRV in patients from the London COPD cohort using paired and unpaired analysis, in the stable state and at natural COPD exacerbation using real-time qPCR.

3.2 Characteristics of patients in unpaired analysis

A total of 58 stable state samples and 279 exacerbation samples from 146 COPD patients were analysed between January 2008 and March 2014 as part of the sample collection performed in the London COPD cohort. The baseline clinical characteristics of the 146 patients included are shown in Table 3.1 below.

FEV ₁ , litres	1.18 (\pm 0.5)
FVC, litres	2.56 (\pm 0.9)
FEV ₁ /FVC, %	46.1 (\pm 14.8)
Predicted FEV ₁ , %	47.0 (\pm 21.7)
Current Smoker, %	33
Age, years	69.8 (\pm 8.5)
Male gender, %	59

Table 3.1: Baseline clinical characteristics of 146 patients in the London COPD Cohort, who participated in study of HRV presence at COPD exacerbation and in the stable state in unpaired samples. Data are presented as mean (\pm SD) or percentage (%).

3.3 Results – unpaired analysis

3.3.1 HRV prevalence in unpaired analysis

From a total of 58 stable state samples, 10 (17.2%) had HRV detected. Of 279 exacerbation samples, 117 (41.9%) were positive for HRV which was significantly higher than in the stable state ($p=0.001$) (Figure 3.1).

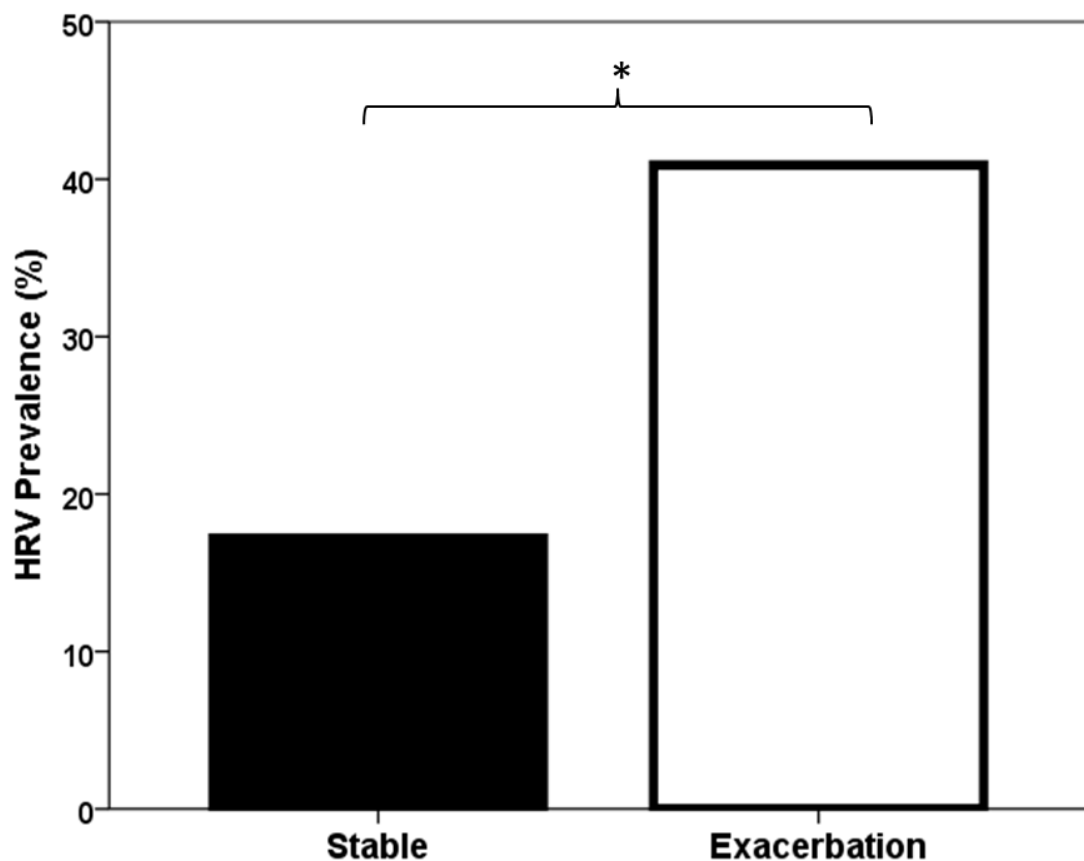


Figure 3.1: Comparison of HRV prevalence in the stable state (n=58) and at COPD exacerbation (n=279) in unpaired samples; * $p=0.001$.

3.3.2 HRV load in unpaired analysis

Specifically examining unpaired samples that were positive for HRV (n=10 in the stable state and n=117 at exacerbation), it was identified that the mean (\pm SEM) HRV load increased significantly at exacerbation ($3.76 (\pm 0.18) \log_{10}$ pfu/ml) compared to the stable state ($2.11 (\pm 0.29) \log_{10}$ pfu/ml); $p=0.008$ (Figure 3.2).

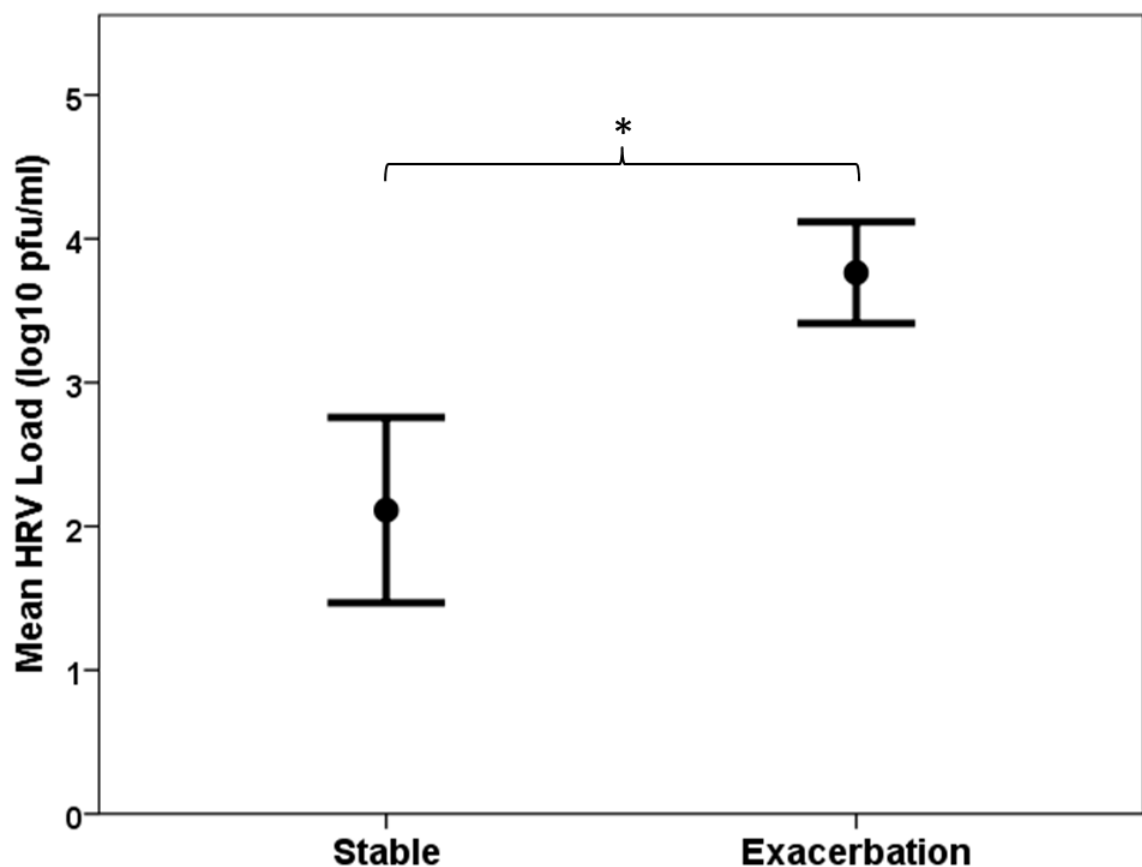


Figure 3.2: Comparison of mean HRV load in the stable state (n=10) and at COPD exacerbation (n=117) in unpaired samples; * $p=0.008$. Data shown as mean (\pm 95% CI).

In absolute terms, there was also a significant increase in the mean HRV load from the stable state to exacerbation (926.25 pfu/ml vs 4.11E+06 pfu/ml; $p=0.042$) (Figure 3.3).

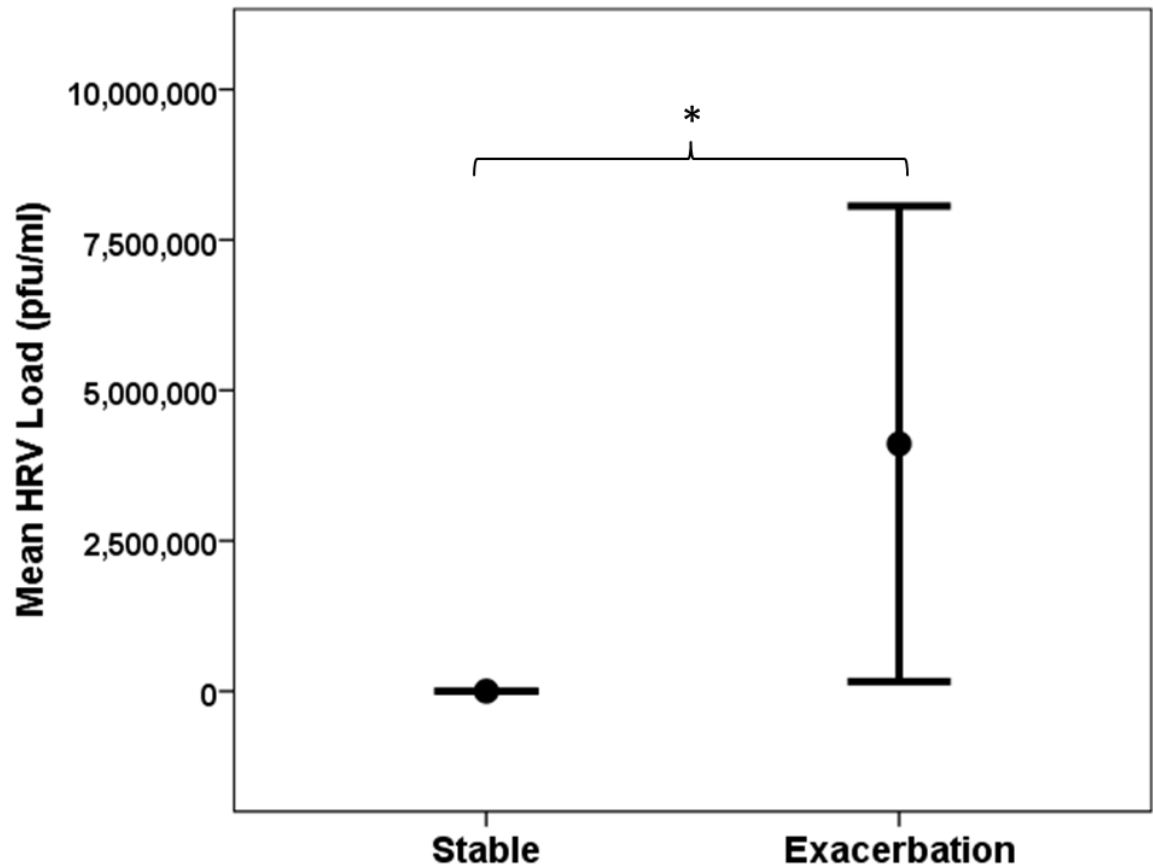


Figure 3.3: Comparison of mean HRV load in HRV-positive samples in the stable state (n=10) and at COPD exacerbation (n=117) in unpaired samples in absolute terms; * $p=0.042$. Data shown as mean ($\pm 95\%$ CI).

3.4 Characteristics of patients in paired analysis

A sub-analysis of 50 COPD patients who had paired stable and exacerbation state sputum samples was performed to reduce any bias that may occur when different patients are used to reflect changes in HRV prevalence and load at different COPD states. Pairs of sputum samples, one taken in the stable state and one at exacerbation were analysed. A total of 54 stable state samples and 54 exacerbation samples from the 50 patients were analysed between January 2008 and December 2012. This sub-analysis involved stable state samples being obtained less than 365 days prior to an exacerbation. The baseline clinical characteristics of these 50 patients included are shown in Table 3.2 below.

FEV ₁ , litres	1.17 (\pm 0.5)
FVC, litres	2.53 (\pm 0.9)
FEV ₁ /FVC, %	46.1 (\pm 14.9)
Predicted FEV ₁ , %	46.7 (\pm 20.7)
Current Smoker, %	35
Age, years	69.4 (\pm 8.5)
Male gender, %	60

Table 3.2: Baseline clinical characteristics of 50 patients in the London COPD Cohort, who participated in a study of HRV presence at COPD exacerbation and in the stable state in paired samples. Data are presented as mean (\pm SD) or percentage (%).

3.5 Results – paired analysis

3.5.1 HRV prevalence in paired analysis

From a total of 54 stable state sputum samples, it was found that 9 (16.7%) were positive for HRV. Of 54 exacerbation samples, 37 (68.5%) showed such presence demonstrating a significantly higher prevalence of HRV at exacerbation compared to the stable state in paired analysis ($p < 0.001$) (Figure 3.4).

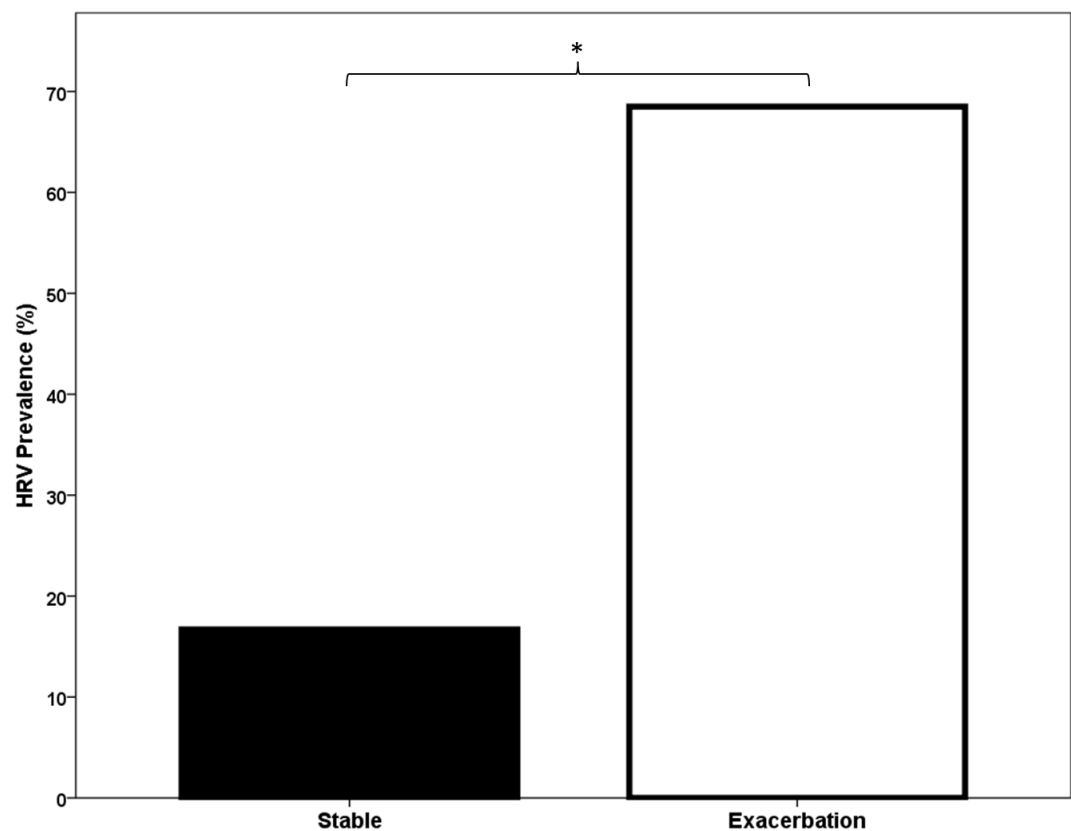


Figure 3.4: Comparison of HRV prevalence in the stable state (n=54) and at COPD exacerbation (n=54) in paired samples; * $p < 0.001$.

Of the 54 pairs of stable state and exacerbation samples, 5 (9.3%) pairs had HRV present at both time points, 32 (59.3%) were positive for HRV at exacerbation only, 4 (7.4%) were positive for HRV in the stable state only and 13 (24.1%) were not positive for HRV at either time point (Figure 3.5). The percentage of pairs with HRV present at only exacerbation was significantly higher than those who had HRV detected in the stable state only, at both disease states and at neither time point (all $p < 0.001$). There was a significantly higher percentage of pairs who did not have HRV detected at either time point compared to the percentage of those who had HRV in the stable state only ($p = 0.017$) or at both disease states ($p = 0.039$).

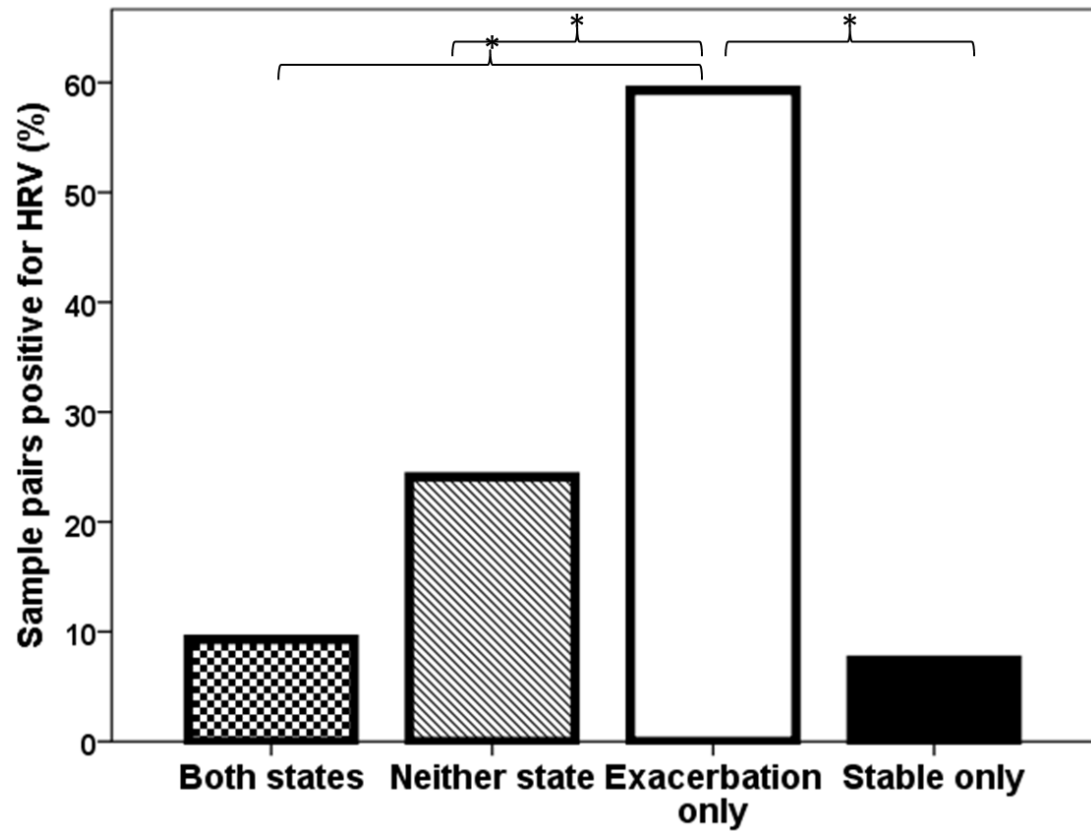


Figure 3.5: Comparison of the percentage of sample pairs (n=54) positive for HRV at exacerbation only, in the stable state only, in the stable state and at exacerbation (both states), and in neither the stable state or at exacerbation (neither state); *p<0.001.

3.5.2 HRV load in paired analysis

The HRV load in HRV-positive samples from patients in whom there was paired stable state and exacerbation samples available, was then compared. The 9/54 samples that were found to be positive for HRV in the stable state had a mean HRV load of $1.96 (\pm 0.27)$ \log_{10} pfu/ml. The 37/54 samples that had HRV detected at exacerbation had a mean load of $3.76 (\pm 0.33)$ \log_{10} pfu/ml which was significantly higher than in the stable state ($p=0.011$) (Figure 3.6).

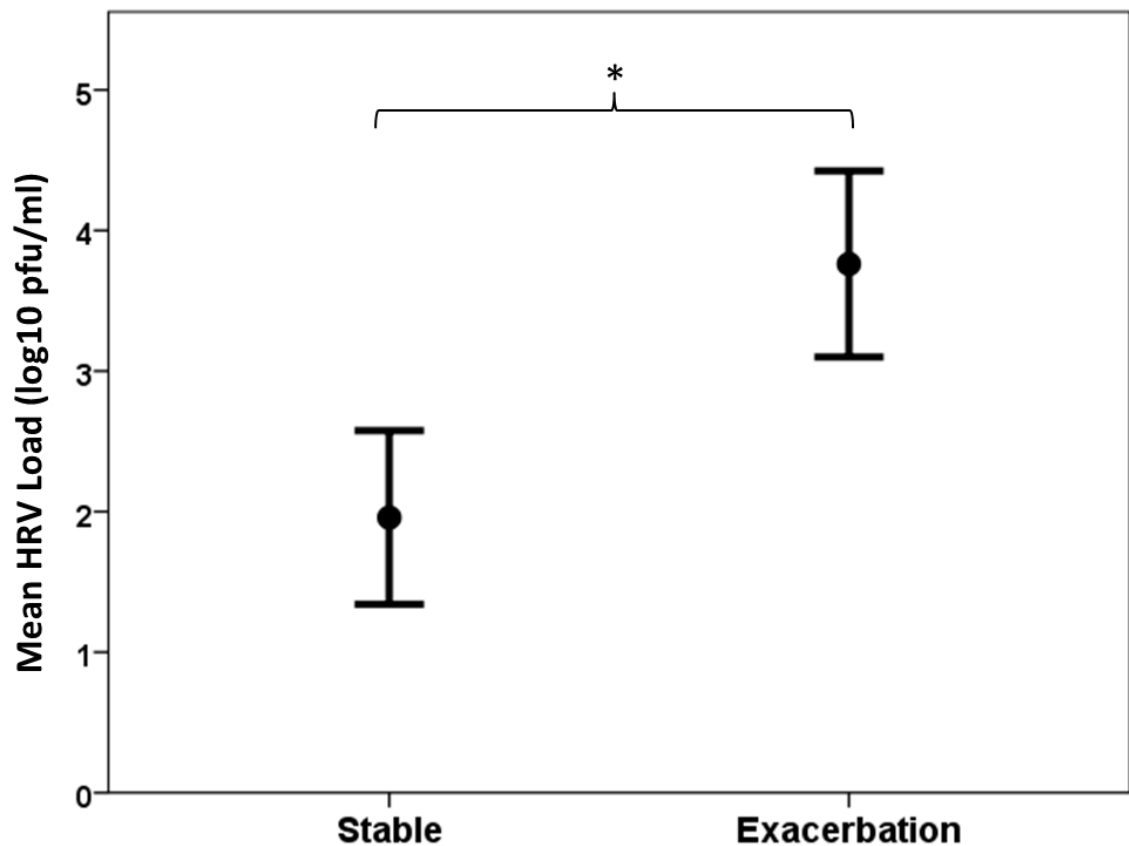


Figure 3.6: Comparison of mean HRV load in HRV-positive samples in the stable state (n=9) and at COPD exacerbation (n=37) in paired samples; * $p=0.011$. Data shown as mean ($\pm 95\%$ CI).

Additionally, it was established that 37 of the 54 pairs (68.5%) had a higher HRV load at exacerbation compared to the stable state, 13 showed no signs of HRV presence at either stable or exacerbation states, and four showed a fall in HRV load from the stable state to exacerbation (Figure 3.7).

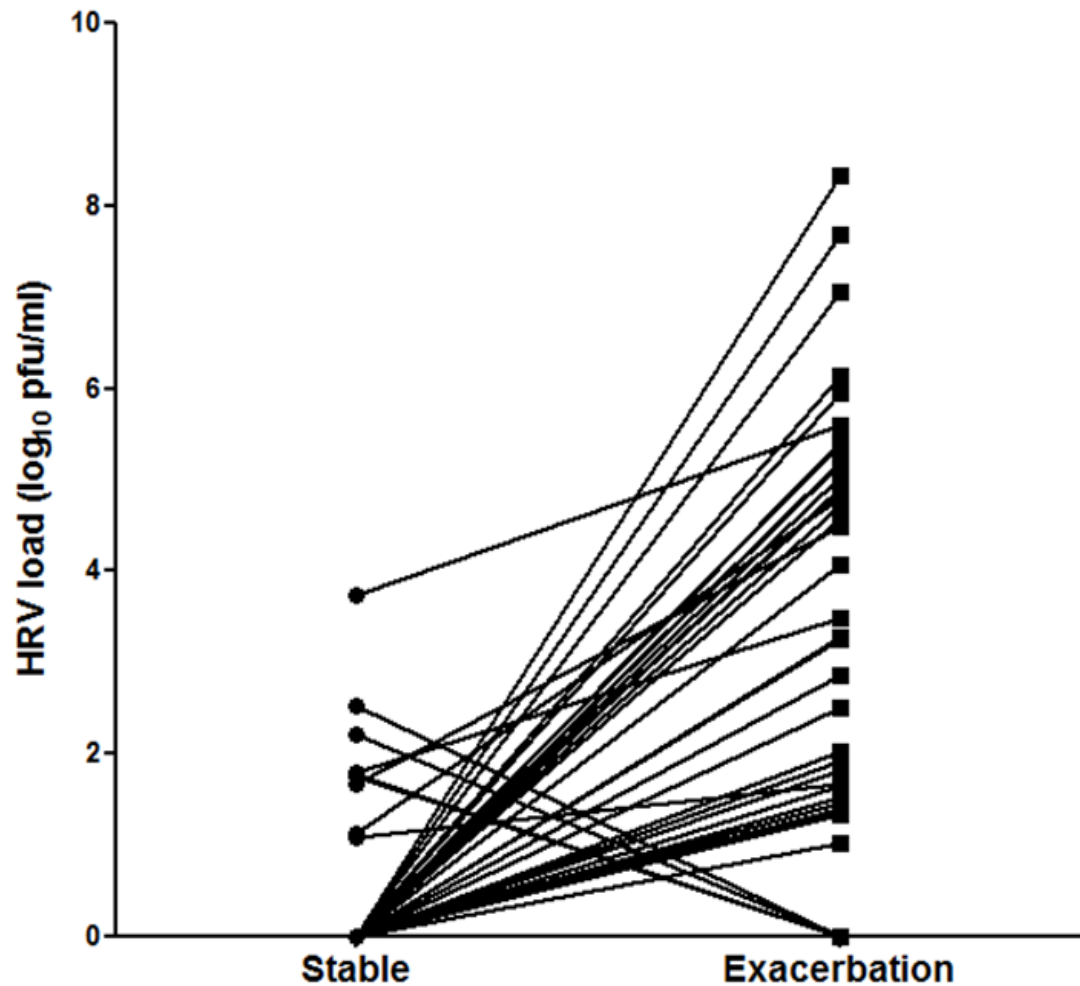


Figure 3.7: Changes in HRV load in 50 patients with paired stable state (n=54) and exacerbation state (n=54) data.

Five of the 54 pairs were HRV-positive at both the stable state and at exacerbation. The HRV load was significantly higher in the exacerbation state ($4.01 (\pm 0.68) \log_{10} \text{ pfu/ml}$) compared to in the stable state ($1.88 (\pm 0.48) \log_{10} \text{ pfu/ml}$); $p=0.034$ (Figure 3.8).

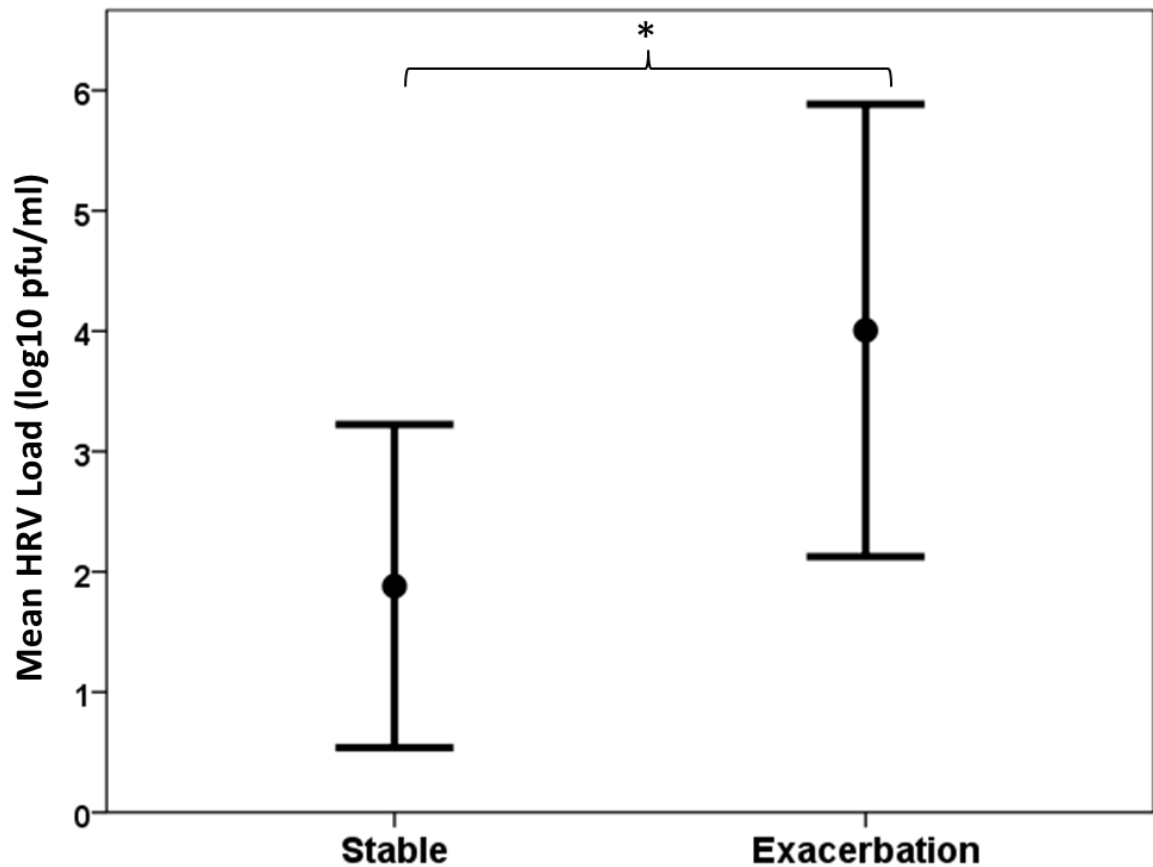


Figure 3.8: Comparison of mean HRV load in the stable state (n=5) and at COPD exacerbation (n=5) in paired samples positive for HRV at both disease states; * $p=0.034$. Data shown as mean ($\pm 95\%$ CI).

3.6 Discussion

This study assessed changes in the prevalence and load of HRV infection in sputum samples from the airways of COPD patients using real-time qPCR in the stable state and at acute exacerbation.

Previous studies exploring viral infection in COPD have shown a higher rate of virus detection at exacerbation compared to stable COPD. Rohde and colleagues detected respiratory viruses in 47% of sputum exacerbation samples which was significantly higher than the 10% detected in stable state sputum (Rohde et al., 2003). However when using nasal samples as opposed to sputum samples, the difference in viral prevalence between the two disease states was not significant (Rohde et al., 2003). Similarly, Liao found that HRV increased significantly from 5.3% in the stable state to 21.9% at exacerbation in sputum samples but from 3.5% to 13.2% in nasal samples (Liao et al., 2014). Tan and colleagues reported an increase in viral detection from 7% in the stable state to 64% at exacerbation (Tan et al., 2003) and Papi and colleagues found 48.4% of exacerbations to be positive for viral infection compared to 6.2% in stable COPD (Papi et al., 2006). In 2001, Seemungal and colleagues reported HRV to be the most prevalent virus detected at COPD exacerbation (Seemungal et al., 2001). Furthermore, this group showed that HRV prevalence increased from 7.4% in the stable state to 59.1% at exacerbation (Seemungal et al., 2000b). The results from the current study are consistent with the findings from

these previous studies showing the prevalence of HRV in sputum to increase significantly from 17.2% in the stable state, to 41.9% at COPD exacerbation.

Compared to the number of studies examining changes in HRV prevalence in COPD, fewer studies have explored changes in HRV load using qPCR techniques however it is important to investigate changes in HRV load as well as prevalence to further understand the role of HRV in COPD. In 2010, Quint and colleagues explored HRV load changes between stable COPD and exacerbation and found a significant increase in HRV load at exacerbation compared to stable state (Quint et al., 2010). In 2011, Mallia and colleagues experimentally infected mild COPD patients with HRV and found that HRV was detected in airway sputum samples following inoculation and that HRV load increased after inoculation, peaking on day 5 (Mallia et al., 2011). The results from the current study found HRV load to be significantly increased at exacerbation compared with stable COPD which is consistent with these previous studies. Furthermore, the load was also found to increase from stable COPD to exacerbation when exploring HRV load in absolute terms. When evaluating HRV load changes in patients with paired stable state and exacerbation state data, it was found that in 68.5% of paired samples there was an increase in HRV load from the stable state to exacerbation suggesting that increases in HRV load detected at COPD exacerbation, are not driven by just a small number of patients, but occur in the majority of cases.

While HRV prevalence and load have been shown to be significantly higher at exacerbation than in the stable state, it is important to consider viral infection in stable COPD. Although HRV was detected in few stable state samples in this sample set, HRV loads were low, which suggests that circulating HRV infection may be present in these patients, however it did not lead to the onset of symptoms or an exacerbation. Furthermore, in some instances, HRV detection in the stable state may be the end product of a previous HRV-associated exacerbation or the start of a new one. In this study however, patients were only considered stable providing there had been no exacerbation onset for four weeks prior to, or two weeks after, the stable state sample being collected.

The main findings from this study confirm the association of HRV infection with COPD exacerbations suggested previously by other groups. The significant increase in HRV load from the stable state to exacerbation indicates that HRV load may be a critical factor in contributing to exacerbations of COPD. Conversely, it must be considered that an exacerbation itself may create an environment which allows HRV to increase in load however, the experimental work by Mallia and colleagues (Mallia et al., 2011) supports the initial suggestion and based on the results of the current study, it seems HRV infection plays a key role in the onset of COPD exacerbations. These findings illustrate the importance of HRV infection at COPD exacerbation and emphasise the need for the development of therapy to treat HRV infections with the aim of reducing exacerbation frequency and improving health status, particularly as it is thought that current

treatments only reduce mortality rates by approximately 25% (TORCH, 2004). The current findings provide further evidence for the role of HRV in COPD exacerbations and should provide a basis for further study into HRV infection in COPD. The findings stress the importance of HRV and emphasise the need for the development of HRV specific antiviral therapy.

HRV infection is a common cause of upper respiratory tract infections (Pattemore et al., 1992) but the ability of HRV to infect the lower airways remained controversial as evidence suggested the optimal growth temperature for most HRV serotype was 33°C which is the temperature of the nasal epithelium (Papadopoulos et al., 2000, 1999). However a number of studies showed that in fact HRV was able to infect the lower airways (Gern et al., 1997; Horn et al., 1979; Liao et al., 2014; Papadopoulos et al., 1999; Seemungal et al., 2000). Liao and colleagues showed a higher prevalence and load of HRV, at both the stable state and at exacerbation, in sputum samples compared to nasal samples (Liao et al., 2014) and Seemungal and colleagues showed a 40% higher prevalence of HRV in induced sputum samples from the lower airways compared to nasopharyngeal samples from the upper airway (Seemungal et al., 2000). A study exploring the role of viruses and bacteria using nasal swabs, sputum and throat swabs found that virus was recovered more often from sputum samples than throat or nasal samples, suggesting that viral replication can occur freely in the lower respiratory tract (Horn et al., 1979). The findings from these studies indicate that the use of sputum is not

only suitable, but preferable, for the detection of viruses so throughout this study sputum samples were used for HRV detection and quantification.

An important component of the data analysis performed in this chapter involved using both paired and unpaired data. Unpaired analysis is more commonly used than paired analysis in studies comparing differences in HRV infection between stable and exacerbation states of COPD (Papi et al., 200; Rohde et al., 2003; Seemungal et al., 2001; Tan et al., 2003) which may be due to the challenges that often occur with longitudinal studies. These difficulties were overcome in the current study and showed significant increases in HRV prevalence and load from stable COPD to exacerbation using both methods of analysis. It must be recognised that paired analysis also introduces its own population bias as only chronic sputum producers are included in this analysis.

Until now, studies of natural exacerbations have focused on the changes in HRV infection in the stable state and at exacerbation. However, in order to advance our understanding of HRV infection in COPD, it is important to explore HRV infection during the time-course of exacerbation and recovery in naturally occurring exacerbations. Furthering the current knowledge of HRV and investigating changes in the time-course of HRV prevalence and load during natural exacerbations will allow the pattern of HRV to be explored leading to potential modifications in the current approaches to COPD patient treatment strategies.

These results may help to provide better strategies for the management of COPD exacerbations and health-care planning. Chapter 4 investigates HRV infection during naturally occurring COPD exacerbation and recovery in patients in the London COPD cohort.

3.7 Conclusion

The findings from this study showed that the prevalence of HRV in sputum was greater at exacerbation than in the stable state. Similarly, HRV load measured by qPCR was significantly higher at exacerbation compared with the stable state in both unpaired and paired analysis. These findings emphasise the importance of HRV infection at COPD exacerbation, and stress the significance of HRV as a target for therapy in COPD with the objective of reducing exacerbation frequency and improving health status.

CHAPTER 4. Time-course of HRV infection during COPD exacerbation and recovery

4.1 Introduction

The current study aimed to explore HRV infection in COPD patients over the entire time-course of natural exacerbations, from exacerbation presentation until Day 56 post-exacerbation. Furthermore, it aimed to examine whether certain inflammatory markers could be used as indicators of HRV infection at various time-points during COPD exacerbation and recovery.

As reported in the previous chapter, human rhinovirus (HRV) prevalence and load were significantly increased in the airways of COPD patients at exacerbation compared to in the stable state. As discussed previously, changes in HRV infection between these two disease states has been explored by a number of studies, however, HRV infection during the entire time-course of COPD exacerbations and recovery, has not yet been investigated in naturally occurring COPD exacerbations.

Experimental HRV infection studies in asthma have yielded important insights into disease mechanisms (Message et al., 2008; Wark et al., 2005) however no similar experimental model existed for COPD until 2006 when Mallia and colleagues performed a pilot investigation in which mild COPD patients were experimentally infected with HRV (Mallia et al., 2006). This work was advanced in 2011 using larger study numbers and explored the changes in HRV load over the time-course of exacerbations and recovery, and found that in sputum samples HRV load was shown to peak at Day 5 post-inoculation with HRV (Mallia et al., 2011). This study is a valid

model of HRV infection over the time-course of COPD exacerbations and recovery using experimental infection, which allows HRV infection to be measured in a controlled manner. However this study only involved mild COPD patients and low dose HRV for inoculation, which does not necessarily give a true representation of natural HRV infection found in COPD patients in the community. It is necessary to investigate HRV changes in subjects with varying degrees of disease severity and with natural, community-acquired HRV infection. The current study which explores HRV infection over the time-course of naturally occurring COPD exacerbations and recovery will provide new insight into the pattern of HRV infection over the time-course that has not been investigated previously. The findings of this study may impact on the way in which HRV-associated exacerbations of COPD are treated clinically, and stimulate further research into HRV infection in natural exacerbations of COPD.

Inflammatory markers are a class of proteins whose levels increase or decrease in response to inflammation. They can be used to give an indication of the aetiology of COPD exacerbations such as whether they are of an infective or non-infective nature, which may impact on exacerbation management. They may also be used to give an indication of exacerbation recovery time and prognosis, however are used more widely as research tools as opposed to being used clinically. Mallia and colleagues found a significant increase in IL-8 levels at Day 9 post-inoculation but saw no change in IL-6 or TNF- α . To date, changes in the levels of IL-6 and IL-8 between stable COPD and exacerbation have been reported; Hurst and colleagues showed that IL-6 levels in plasma were significantly increased from the stable state to exacerbation (Hurst et al.,

2006), and Seemungal and colleagues showed an increase in sputum IL-6 at exacerbation compared to the stable state but no significant changes in IL-8 (Seemungal et al., 2000). Further studies have shown a relationship between changes in inflammatory markers and bacterial or viral infection. It was shown in 2000 that increased airway inflammation was associated with the isolation of certain bacterial species; in particular, exacerbations associated with *H influenzae* were found to have increased levels of IL-8 and TNF- α and those associated with *M catarrhalis* had increased levels of TNF- α (Sethi et al., 2000). Inflammatory markers have also been shown to increase with bacterial load increases, in stable chronic bronchitis patients (Hill et al., 2000). In 2010, Quint and colleagues showed that interferon- γ -inducible protein (IP-10) levels increased significantly from the stable state to exacerbation in COPD patients. IP-10 is a chemokine secreted by bronchial epithelial cells, monocytes and leukocytes in response to interferon- γ (IFN- γ) and TNF- α . The study reported a significant relationship between HRV load and IP-10 levels showing IP-10 to be a successful marker of HRV infection. IL-6 levels were shown not to correlate with changes in HRV load, however increases in IL-6 levels from the stable state to exacerbation were greater in the presence of HRV (Quint et al., 2010). Saldias and colleagues showed a significant increase in IL-6, IL-8 and TNF- α at exacerbations with IL-6 increasing particularly in the presence of airway bacteria. Compared to viral exacerbations, bacterial-associated exacerbations were associated with a higher degree of systemic inflammation (Saldías et al., 2012). Wilkinson and colleagues also showed an increase in IL-8 levels in bacterial exacerbations and showed that patients with more severe COPD exhibited a greater rise in inflammation at exacerbation

compared to more mild patients (Wilkinson et al., 2006). These studies illustrate how certain inflammatory markers increase at COPD exacerbation and in the presence of infection. They have been shown to be important in terms of indicating exacerbation onset by increasing significantly from the stable state to exacerbation. To date however, the changes in levels of inflammatory markers have not been investigated over the recovery period in naturally occurring exacerbations association with HRV infection.

This study aimed to explore the prevalence and load of HRV over the time-course of natural exacerbations, from exacerbation presentation until Day 56 post-exacerbation and also to examine the potential use of inflammatory markers as indicators of HRV infection at various time-points during COPD exacerbation and recovery.

4.2 Characteristics of patients in unpaired analysis

A total of 537 samples from 71 COPD patients were analysed between April 2010 and June 2013 as part of the sample collection performed in the London COPD cohort. These 537 samples were taken at 6 different time-points during exacerbation and recovery; 129 samples were taken at exacerbation presentation (Exp), 92 at Day 3 post-exacerbation, 115 at Day 7, 102 at Day 14, 77 at Day 35 and 22 at Day 56. The baseline clinical characteristics for these patients are shown in Table 4.1.

FEV ₁ , litres	1.19 (±0.5)
FVC, litres	2.45 (±0.8)
FEV ₁ /FVC, %	46.2 (±14.9)
Predicted FEV ₁ , %	45.9 (±19.6)
Current Smoker, %	33
Age, years	72 (±8.0)
Male gender, %	61

Table 4.1: Baseline clinical characteristics of 71 patients in the London COPD Cohort, who participated in a study of HRV presence during COPD exacerbation and recovery in unpaired analysis. Data are presented as mean (±SD) or percentage (%).

4.3 Results – unpaired analysis

4.3.1 Change in HRV prevalence over time

From a total of 129 exacerbation presentation (ExP) samples, 46.5% (60/129) were found to be positive for HRV. Of the 92 samples taken at Day 3, 30.4% (28/92) were positive for HRV, 23.5% (27/115) at Day 7, 13.7% (14/102) at Day 14, 5.2% (4/77) at Day 35 and 4.5% (1/22) at Day 56. There was a significant fall in the prevalence of HRV from ExP to each time point during the recovery period (all $p < 0.014$). There were also significant decreases in HRV prevalence from Day 3 to Day 14 ($p = 0.004$), Day 35 ($p < 0.001$) and Day 56 ($p < 0.001$), and from Day 7 to Day 35 ($p = 0.001$) and Day 56 ($p = 0.044$) (Figure 4.1).

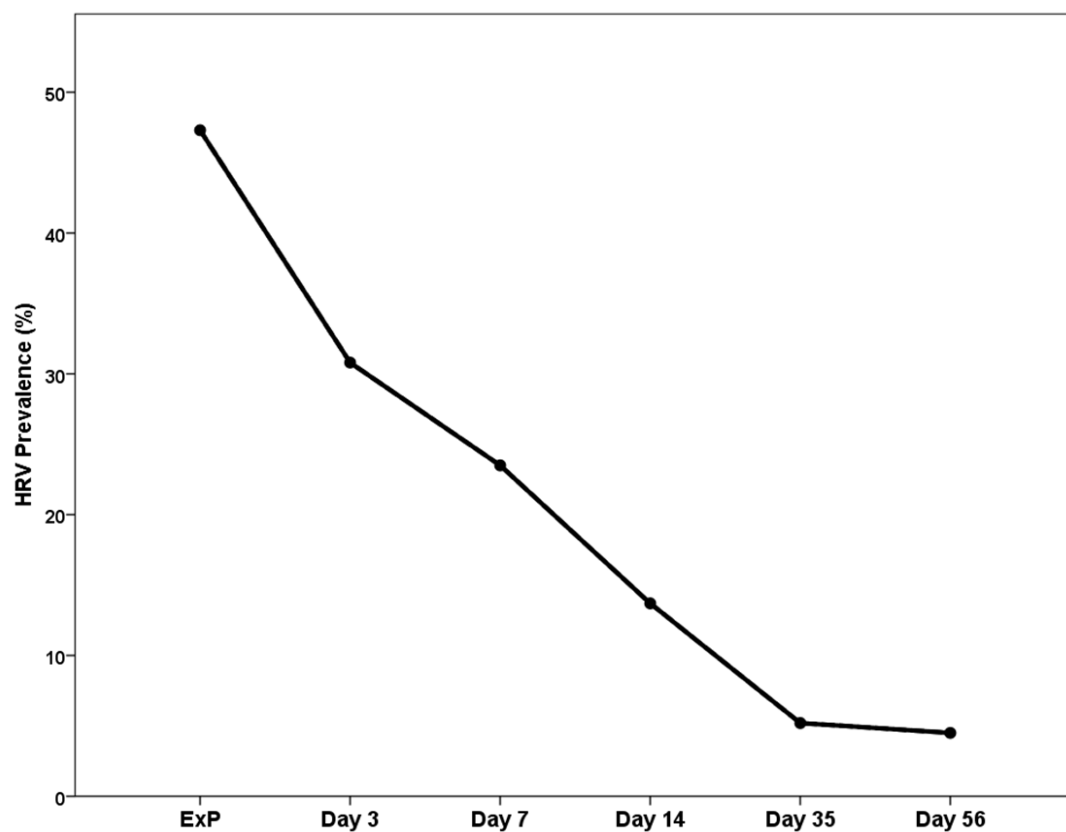


Figure 4.1: Change in the HRV prevalence during the time-course of COPD exacerbations from presentation (ExP) and during recovery in unpaired analysis.

4.3.2 Change in HRV load over time

In the exacerbations positive for HRV at presentation (ExP) (n=60), the median (IQR) HRV load decreased significantly from ExP ($10^{3.27(1.68-4.90)}$ pfu/ml) to Day 3 ($10^{1.29(0-2.56)}$ pfu/ml), to Day 7 ($10^{0(0-1.67)}$ pfu/ml), to Day 14 ($10^{0(0-0)}$ pfu/ml), to Day 35 ($10^{0(0-0)}$ pfu/ml) and to Day 56 ($10^{0(0-0)}$ pfu/ml) (all $p < 0.001$). There was also a significant fall in median HRV load from Day 3 to Day 14, Day 35 and Day 56 (all $p < 0.001$) and from Day 7 to Day 35 and Day 56 (both $p \leq 0.003$) (Figure 4.2).

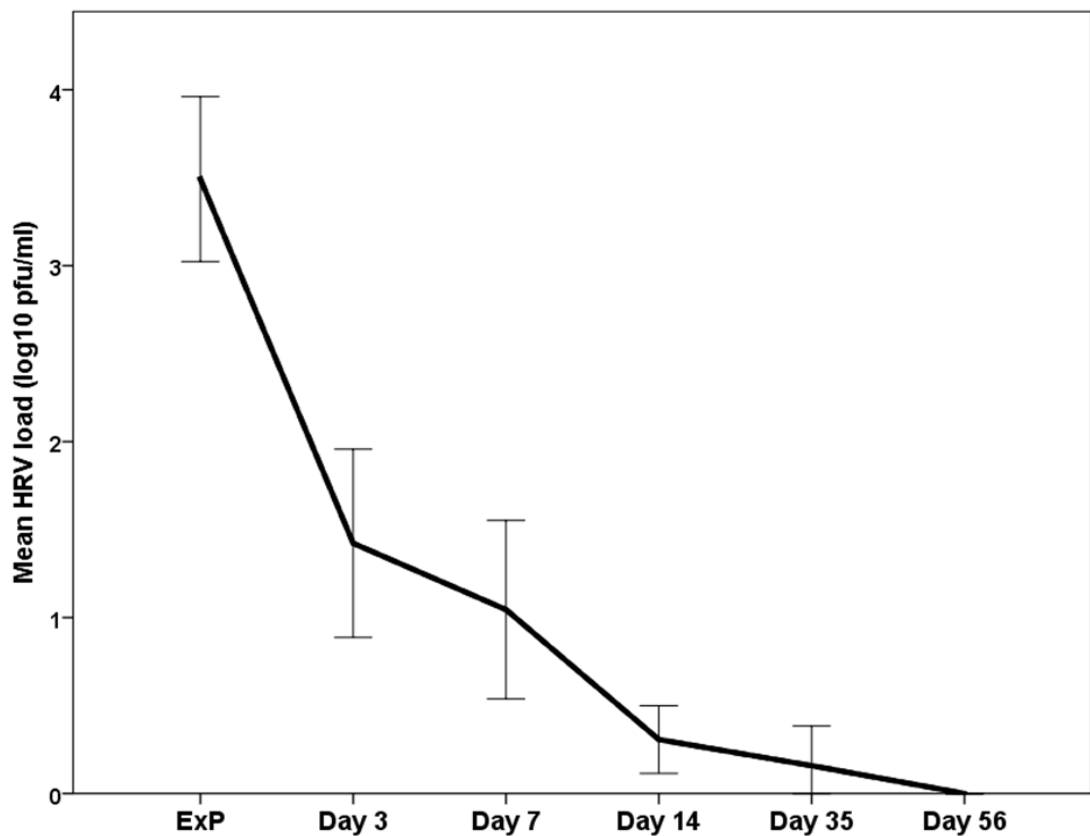


Figure 4.2: Change in the HRV load during the time-course of COPD exacerbations from presentation (ExP) and during recovery in unpaired analysis. Data shown as mean ($\pm 95\%$ CI).

4.3.3 Treatment

Of the 129 exacerbations, 93 (72.1%) were treated with corticosteroids at presentation; 91.1% of these for 7 days of corticosteroids treatment. There was no difference in the HRV load at any time point, from ExP to Day 56, between exacerbations treated with corticosteroids compared to those not treated. Similarly with antibiotics, of the 129 exacerbation, 122 (94.6%) were treated with antibiotics at presentation; 92.6% of these for 7 days. There was no difference in HRV load at any time point, from ExP to Day 56, between exacerbations treated with antibiotics and those that were not.

4.4 Characteristics of patients in paired analysis

In a sub-analysis of 22 patients, paired sputum samples i.e. samples taken at each of the 5 time-points from exacerbation presentation (ExP) to Day 35 post-exacerbation were analysed (there was only a small number of patients who attended the Day 56 post-exacerbation visits) over 28 exacerbations. This paired analysis aimed to reduce or avoid any bias that may occur when different patients are used to show changes and differences in HRV infection at different during COPD exacerbation and recovery.

For the paired analysis, there were 140 samples from these 22 patients; 28 samples were taken at each of the five time-points from ExP and during recovery until Day 35.

The baseline clinical characteristics for these 22 patients are shown in Table 4.2.

FEV ₁ , litres	1.15 (\pm 0.5)
FVC, litres	2.29 (\pm 0.9)
FEV ₁ /FVC, %	46.2 (\pm 14.9)
Predicted FEV ₁ , %	44.9 (\pm 20.3)
Current Smoker, %	35
Age, years	70.4 (\pm 8.0)
Male gender, %	61

Table 4.2: Baseline clinical characteristics of 22 patients in the London COPD Cohort, who participated in a study of HRV presence during COPD exacerbation and recovery in paired analysis. Data are presented as mean (\pm SD) or percentage (%).

4.5 Results – paired analysis

4.5.1 Change in HRV prevalence over time

From a total of 140 samples, 28 were taken at each time point from ExP until Day 35 post-exacerbation. Of the 28 samples taken at ExP, 53.6% (15/28) were found to be positive for HRV. At Day 3 the HRV prevalence was 42.9% (12/28), 35.7% (10/28) at Day 7, 14.3% (7/28) at Day 14 and 3.6% (1/28) at Day 35. There was a significant fall in the prevalence of HRV from ExP to Day 14 ($p=0.002$) and Day 35 ($p<0.001$) post-exacerbation. There were also significant decreases in HRV prevalence from Day 3 to Day 14 ($p=0.018$) and Day 35 ($p<0.001$), and from Day 7 to Day 35 ($p=0.002$) (Figure 4.3).

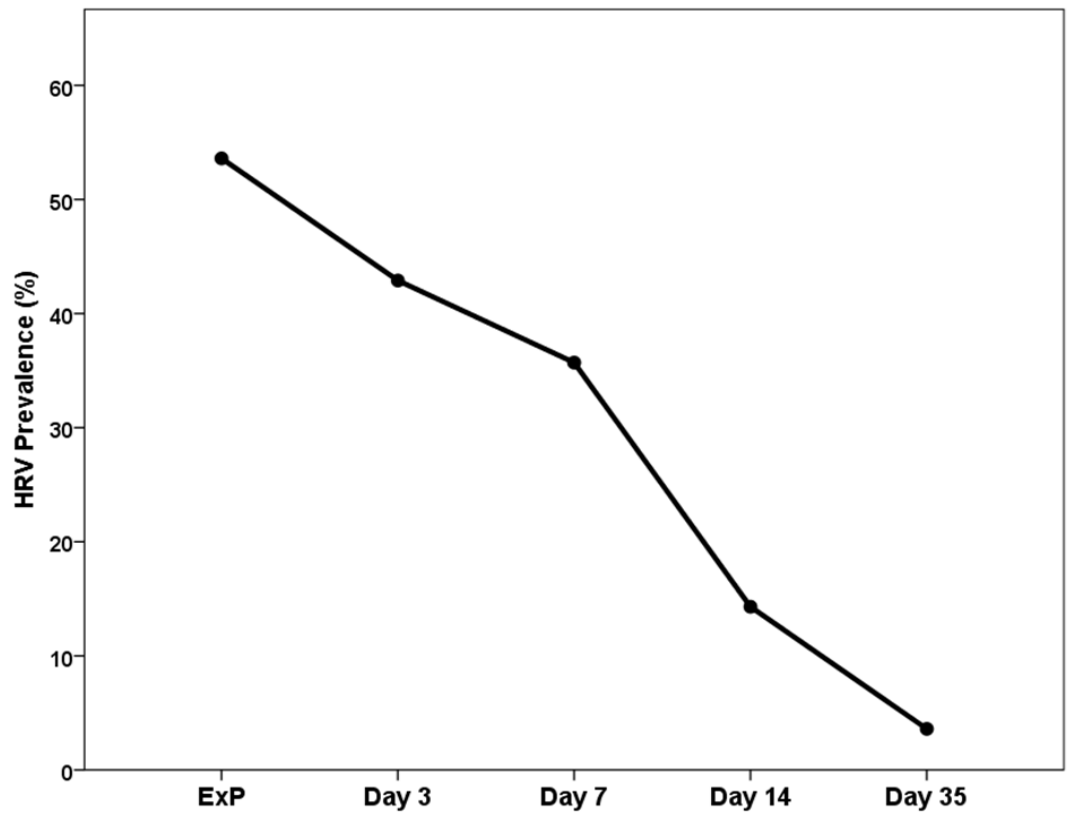


Figure 4.3: Changes in the HRV prevalence during the time-course of COPD exacerbations from presentation (ExP) and during recovery, in paired analysis.

4.5.2 Change in HRV load over time

In exacerbations positive for HRV at ExP (n=15) in this paired analysis, the median (IQR) HRV decreased significantly from ExP ($10^{2.51(1.72-3.88)}$ pfu/ml) to Day 3 ($10^{1.42(0-2.05)}$ pfu/ml) ($p=0.043$) as well as to Day 7 ($10^{0(0-1.37)}$ pfu/ml), Day 14 ($10^{0(0-1.48)}$ pfu/ml) and Day 35 ($10^{0(0-1.48)}$ pfu/ml) (all $p<0.002$). There was also a significant decrease in the median HRV load from Day 3 to Day 7, from Day 7 to Day 14 and from Day 14 to Day 35 (all $p<0.001$) post-exacerbation (Figure 4.4).

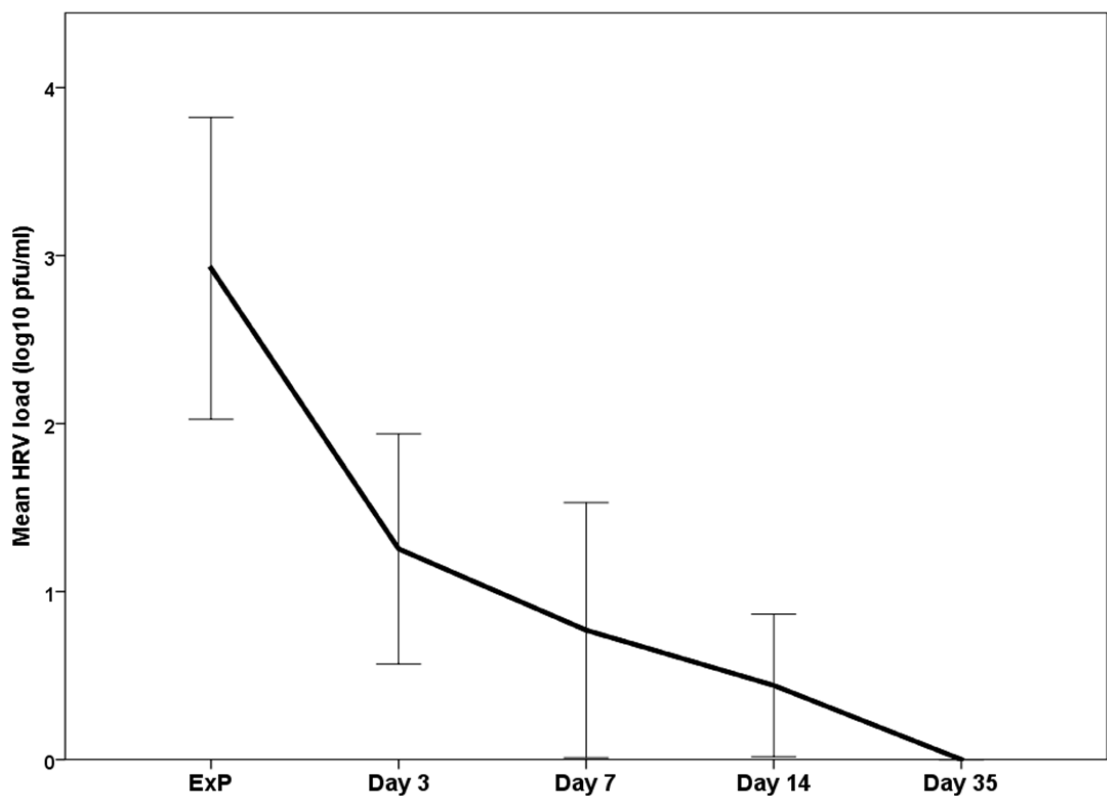


Figure 4.4: Change in the HRV load during the time-course of COPD exacerbations from presentation (ExP) and during recovery in paired analysis. Data shown as mean ($\pm 95\%$ CI).

4.5.3 Treatment

Of the 28 exacerbations investigated in this paired analysis, 23 (82.1%) were treated with corticosteroids at presentation; 95.7% of these for 7 days. There was no difference in the HRV load at any time point, from ExP to Day 35, between exacerbations treated with corticosteroids compared to those not treated. Of the 28 exacerbations, 100% were treated with antibiotics at presentation, 92% were treated for 7 days.

4.6 HRV infection and inflammatory markers

Data was available for inflammatory markers IL-6, IL-8, IL-1 β , IL-18, TNF- α , CRP, fibrinogen and IFN- γ at all time-points during the exacerbation and recovery period, from ExP (n=105) until Day 35 post-exacerbation.

Inflammatory marker levels were investigated in two groups; exacerbations positive for HRV infection and exacerbations negative for HRV infection. At Day 7, the levels of sputum IL-6 were significantly higher in the presence of HRV compared to without HRV ($p=0.014$) (Figure 4.5D) but no difference was seen in IL-6 levels at any other time points. At Day 14, the levels of IFN- γ were higher in the presence of HRV compared to without HRV ($p=0.048$) (Figure 4.5C) but no differences were seen at any other time point. There was no significant difference between the levels of any of the other inflammatory markers measured, in the presence of HRV compared to without HRV, at any other time point (Figure 4.5).

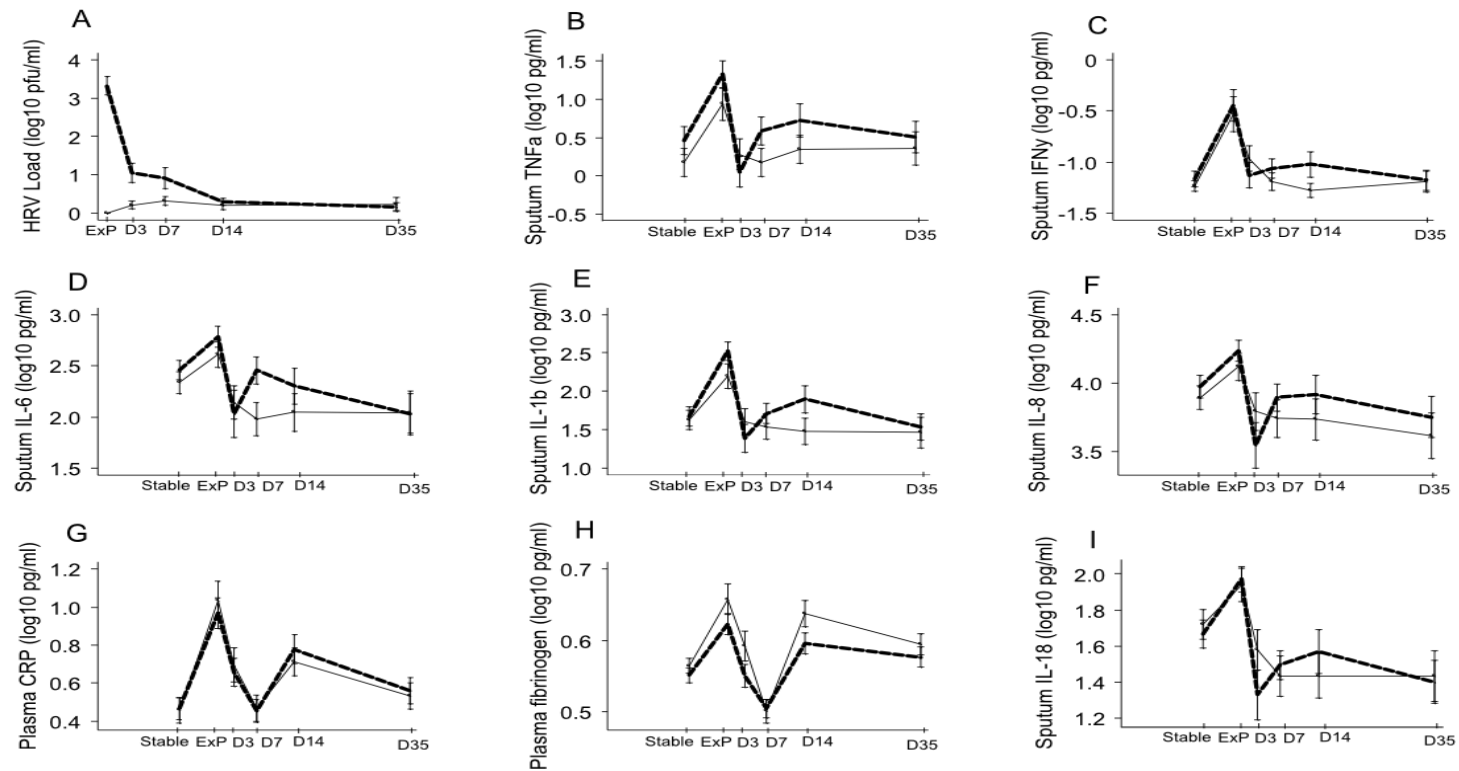


Figure 4.5: Changes in inflammatory marker levels in the stable state, exacerbation presentation (ExP) and during recovery in samples positive for HRV (thick dotted line) and samples negative for HRV (thin solid line). A) HRV, B) TNF- α , C) IFN- γ , D) IL-6, E) IL-1 β , F) IL-8, G) CRP, H) fibrinogen and I) IL-18. Data shown as mean (\pm 95% CI).

4.7 Discussion

For the first time, this study has explored changes in HRV infection over the entire time-course of COPD exacerbation and recovery period using both unpaired and paired analyses. The prevalence and load of HRV was measured at 6 time-points from exacerbation presentation through to Day 56 post-exacerbation. It was shown in the previous chapter (Chapter 3) that HRV prevalence and load increased significantly at COPD exacerbation compared to the stable state, however until now, changes in HRV prevalence and load from exacerbation presentation, and during the recovery period, had not been investigated in natural COPD exacerbations.

It is impossible to compare the results of the current study with any other of its kind as this is novel work that has only been explored previously using experimental infection. As discussed previously, the changes in HRV load during exacerbation and recovery have been explored using experimental models of HRV infection where Mallia and colleagues showed that HRV load was highest at COPD exacerbation and then decreased over the recovery period in mild COPD patients who were experimentally infected with HRV (Mallia et al., 2011). The current study found similar results to the Mallia work when exploring natural HRV infection in COPD exacerbations. It was found that both the prevalence and load of HRV were highest at exacerbation presentation and decreased over the time-course with significant falls in the load from presentation

to Day 3 post-exacerbation and from Day 3 to Day 7 until no HRV was detected by Day 56. HRV prevalence and load were shown to be highest at exacerbation presentation suggesting that patients may become infected with HRV a few days before presenting to the clinic which highlights the importance of early sampling of COPD patients to capture an exacerbation as early as possible. This is also important in terms of exacerbation treatment with the aim of treating patients as early as possible at exacerbation onset to try and reduce exacerbation symptoms and length. Furthermore, finding HRV prevalence and load to be highest at presentation is important, as one of the many obstacles in the development of antiviral therapies is that it is considered that patients are likely to present with symptoms, and be prescribed therapy, too late for the antiviral to have an effect. These findings however illustrate that HRV load is highest when patients report to the clinic suggesting that viral replication may be ongoing and therefore antiviral therapy may be effective at this time point. This highlights the need for the rapid development of antiviral therapies for the treatment of COPD exacerbations as patients have highest HRV loads at exacerbation presentation.

At present there are no antivirals in use for the treatment of HRV in COPD. The development of an antiviral is essential for patients with HRV-associated exacerbations of COPD however until this need is met, the development of guidelines for clinicians to aid treatment in this sub-population of COPD patients may be of use. The findings from

this study add to the knowledge of the natural history of these common and important events. The results give further insight into the pattern of HRV infection and may be useful in helping clinicians counsel and educate patients about HRV-associated exacerbations. Furthermore, focusing on natural HRV infection and natural exacerbations allows the generation of genuine results based on COPD patients in the community. This work allows further investigation into the mechanisms of HRV infection and HRV-induced exacerbations of COPD and should stimulate research into novel antiviral therapies for HRV-infected COPD patients. Although there have been a number of molecules that have demonstrated activity against HRV serotypes *in vitro*, these have not been developed for use in humans. This is mainly due to the lack of mouse models for HRV therapy research. The majority of HRV serotypes are part of the major group of HRVs which bind to human ICAM-1 receptors in the nasal epithelium and therefore cannot infect mice. A study that developed a transgenic mouse that expressed human ICAM-1 found that infected mice had increased chemokines, cytokines, neutrophils and viral RNA (Bartlett et al., 2008). When using an anti-ICAM-1 antibody in the mouse model, there was a decrease in inflammatory cells and cytokines and a significant reduction in HRV replication was shown (Traub et al., 2013). Furthermore, a study exploring highly conserved regions of the HRV proteome, in the mouse model, with the aim of finding them to be useful candidates for a broadly cross-reactive vaccine, found that conserved regions of the HRV capsid induced cross-reactive immune responses and were therefore potential candidates for use in a

subunit vaccine (Glanville et al., 2013). These studies were the first to successfully demonstrate a therapeutic intervention in a small animal model of HRV infection which provides optimism for the prospect of testing potential compounds *in vitro* with the possibility of use in humans (Gunawardana et al., 2014).

Previous studies have shown changes in the levels of inflammatory markers between stable state COPD and exacerbation (Bhowmik et al., 2000; Hurst et al., 2006; Quint et al., 2010); the time-course of an exacerbation and recovery has been explored to a lesser extent at present. The levels of IL-8 have been shown to increase in the sputum of experimentally infected COPD patients, peaking on Day 9 post-inoculation. However no significant changes were found in other inflammatory markers such as IL-6 and TNF- α (Mallia et al., 2011). To date, there have been no studies investigating the changes in the levels of inflammatory markers over the time-course of an exacerbation and recovery, with natural HRV infection, in particular comparing the levels of inflammatory markers in the presence of HRV infection and in the absence of HRV infection. The current study is the first to explore this, focusing on the inflammatory markers IL-6, IL-8, IL-1 β , IL-18, TNF- α , CRP, fibrinogen and IFN- γ . This panel of inflammatory markers were chosen as the best evidence in the literature at the time suggested they would be useful (Footitt et al., 2013; Makarova et al., 2013; Mallia et al., 2011; Piper et al., 2013). The current study showed that at Day 14 post-exacerbation, the levels of IFN- γ were significantly higher in the presence of HRV compared to without HRV, and at Day 7 IL-6

levels were significantly higher in the presence of HRV compared to without HRV. However other than these differences, there were no significant differences in the levels of any of the inflammatory markers measured, at any time point, between the two groups. IFN- γ has been shown to be a key constituent of innate immune responses to viral infection by the production of interferons (IFN) by infected cells (Mallia et al., 2011). It has been shown that primary airway epithelial cell cultures from COPD patients demonstrated increased expression of IFN in response to HRV infection (Schneider et al., 2010). It has also been shown that levels of IFN- β are lower in HRV-infected COPD subjects compared to HRV-infected healthy controls which suggests that deficient IFN- β production may contribute to increased susceptibility to HRV infection in COPD (Mallia et al., 2011). Furthermore, Becker and colleagues showed how IFNs significantly reduced HRV replication after high- and low-dose inoculation of primary human bronchial epithelial cells suggesting that exogenous IFNs warrant further study as potential therapy for asthma exacerbations (Becker et al., 2013), and Djukanovic and colleagues showed that inhaled IFN- β may be used as a potential treatment for virus-induced deteriorations in asthma (Djukanović et al., 2014). This may be applicable to COPD also. Although IFNs have been shown to play a role in HRV infection in terms of reducing HRV infection or as potential therapy, the results from the current study suggest that IFN- γ does not reflect HRV infection at exacerbation or during recovery except at Day 14 when HRV load had significantly decreased compared to exacerbation presentation, and is therefore not a good enough indicator of changes in HRV infection

during exacerbation time-course. IL-6 levels were only shown to be significantly higher in HRV infected samples compared to those not infected with HRV at Day 7 post-exacerbation. One study showed elevated IL-6 levels to be associated with HRV infection at exacerbation (Seemungal et al., 2000) however other studies have shown this not to be the case; Quint and colleagues found no relationship between IL-6 levels and HRV loads in sputum (Quint et al., 2010) as did the work by Mallia and colleagues who found no change in baseline IL-6 levels after infection with HRV (Mallia et al., 2011). Although the current study found higher levels of IL-6 and IFN- γ in the presence of HRV, this was only at Day 7 and Day 14, respectively, when HRV load had fallen significantly from presentation which suggests that the increased levels of inflammatory markers at these time points, may be due to other sources such as the development of secondary bacterial infection or another viral infection. These findings suggest that none of the inflammatory markers measured are representative enough of HRV infection over the time-course and therefore cannot be used as suitable or useful markers of HRV infection. Further study into associations between HRV infection and inflammatory marker levels must be performed before these inflammatory markers, or others, can be considered as indicators of HRV infection.

The limitations of this study may have impacted on the findings. The number of samples may have played a role, where this part of the study may have been underpowered for any significant associations with inflammatory markers, to be

identified. However by repeating the study with higher study numbers, or performing further investigations with other inflammatory markers, more definitive conclusions may be made. Furthermore, there may be a threshold value for HRV to be associated with greater inflammation which was not reached in this study. These limitations are likely to have impacted on the study findings, and further study is required, particularly into IL-6 and IFN- γ , before any true associations between HRV infection and these inflammatory markers during exacerbation recovery, can be determined. Associations between upper respiratory tract (URT) symptoms and HRV infection have been discussed in Chapter 5, in which the data suggests URT symptoms may be a better indicator of HRV infection than the inflammatory markers measured in this study.

In a small number of the time-courses used in this study, it was not possible to obtain data from every patient at every time point during the whole time-course. In some cases patients were unable to attend one or more of their clinic visits during the time-course due to various reasons. In other cases, patients may have attended each of their recovery clinic visits, however were unable to produce sputum at certain visits. This resulted in missed sample collection at some time-points which led to missing data for some patients. Unpaired analysis was used for these time-courses however to minimise bias within the data analysis, paired analysis was also used for the time-courses that had available data for each of the six time-points throughout an exacerbation.

4.8 Conclusion

For the first time, this study explored the changes in HRV prevalence and load during the entire time-course of naturally occurring exacerbations and recovery. HRV prevalence and load was highest at exacerbation presentation and decreased over the time-course of recovery until it was undetectable by Day 56. There was no difference in the levels of inflammatory markers in the presence of HRV compared to the absence of HRV over the time-course, except for IFN- γ at Day 14 and IL-6 at Day 7 which were higher in the presence of HRV compared to without HRV. This suggests that these particular inflammatory markers, measured in this study, are not useful markers of HRV infection during exacerbation and recovery, and further study is required. The findings of this study may have implications in terms COPD exacerbation treatment and should stimulate research into novel antiviral therapies. The results emphasise the importance of rapid development of therapies for HRV with the aim of prescribing antiviral therapy early at exacerbation onset thus reducing exacerbation length and severity.

CHAPTER 5. HRV infection and upper respiratory tract symptoms in patients with COPD

5.1 Introduction

This study aimed to determine the association of cold symptoms and sore throats with both the prevalence and load of HRV, in the stable state, at COPD exacerbation and during the recovery period, in naturally occurring exacerbations.

Relationships between viral infection and upper respiratory tract (URT) symptoms such as cold symptoms and sore throats, at exacerbation, have been reported by a number of studies. In 2001, Seemungal and colleagues investigated the relationship between exacerbations of COPD and viral infection by looking at various parameters including symptoms. The study showed that cold symptoms and sore throats were associated with viral detection at COPD exacerbation, and these symptoms were more strongly associated with viral infection than all other symptoms measured although associations between symptoms and viral quantities was not explored (Seemungal et al., 2001) (Table 5.1).

Symptoms at presentation	Odds ratio	p value
Colds*	3.55	<0.001
Increased dyspnoea and colds*	3.27	0.001
Sore throat	2.27	0.043
Increased sputum volume	1.59	0.182
Increased dyspnoea	1.38	0.42
Increased sputum purulent	1.31	0.421
Increased cough	1.22	0.552
Increased wheeze	1.09	0.786

Table 5.1: Effect of symptoms on detection of viruses in nasal samples during exacerbations of COPD. *Colds; increased nasal congestion and/or increased rhinorrhoea. Adapted from (Seemungal et al., 2001).

A study in 2012 comparing host, virological and environment factors associated with symptomatic and asymptomatic HRV infection found that 35% of HRV symptomatic subjects had reported cold symptoms (Gandhi et al., 2012). A study exploring HRV load in nasopharyngeal samples from immunocompetent and immunocompromised subjects, found that HRV loads $>10^5$ RNA copies/mL were frequently associated with the presence of clinical symptoms in the lower or upper respiratory tract, whereas HRV loads $<10^5$ RNA copies/mL were infrequently associated with clinical symptoms (Gerna et al., 2009). It has

also been reported that viral-associated exacerbations have a longer recovery time in terms of symptoms when compared to non-viral associated exacerbation (Seemungal et al., 2001). Seemungal and colleagues reported the median symptom recovery time of viral-associated exacerbations to be 13 days which was significantly longer than the typical 6 day recovery time seen in non-viral associated exacerbations ($p=0.006$) (Seemungal et al., 2001).

Although there have been studies investigating upper respiratory tract (URT) symptoms and viral prevalence, associations between URT symptoms and viral load, in particular HRV load, have not yet been explored in COPD. Furthermore, the association of URT symptoms and viral infection has been explored at COPD exacerbation but not during the time-course of an exacerbation and recovery period in natural exacerbations. Experimental studies have explored the time-course of URT symptoms with HRV infection; Mallia and colleagues found an increase in URT daily symptom scores in patients with mild COPD experimentally infected with HRV peaking within a week post-inoculation (Mallia et al., 2011). Similarly, a study by Grünberg and colleagues in which non-smoking asthma patients were experimentally infected with HRV or placebo, explored the changes in cold symptoms (sore throat, headache, nasal discharge, stuffy nose, malaise, cough and fever) over the time-course of HRV infection. They showed that in the HRV infected group, the cold symptoms started increasing from Day 0 onwards, peaking on Day 2 and then

returning to baseline by Day 7 (Figure 5.1) and this increase was significantly different from the placebo group ($p < 0.01$) (GRÜNBERG et al., 1999).

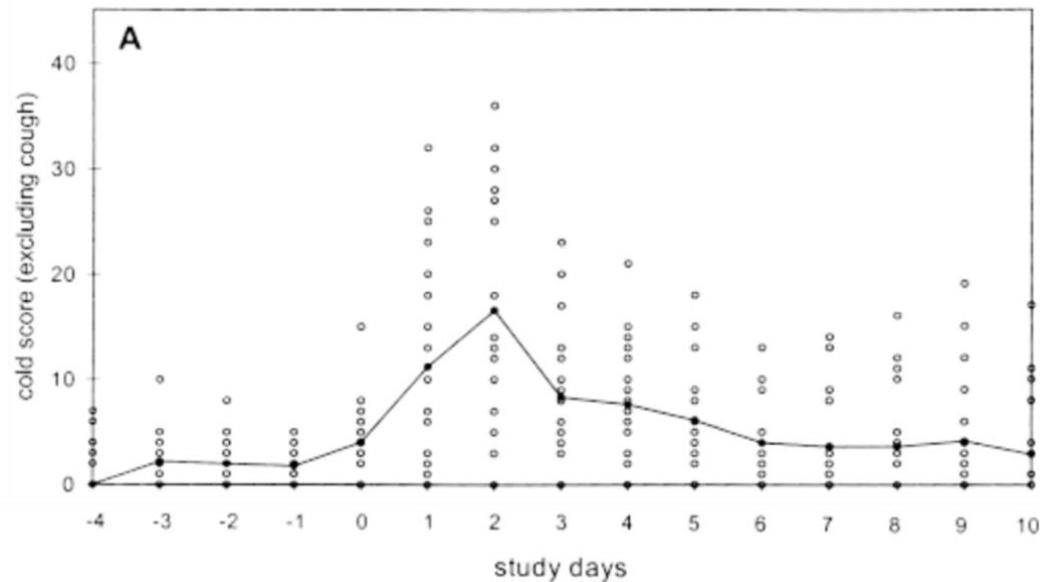


Figure 5.1: Cold symptoms (excluding cough score) in asthmatic patients experimentally infected with HRV. The closed symbols indicate the mean values (GRÜNBERG et al., 1999).

Although there have been experimental studies into associations between URT symptoms and HRV infection during exacerbation and recovery (Mallia et al., 2011), there has not yet been any investigations into URT symptoms and HRV infection over the time-course of naturally occurring exacerbations and recovery period. Additionally, in the majority of studies in this subject, cold symptoms and sore throats have been examined collectively rather than as separate symptoms. However, comparisons between HRV infection in patients reporting one symptom and those reporting multiple symptoms have not been

explored. This may be a useful area to explore if the number of symptoms experienced by a patient is related to HRV prevalence or load which may therefore impact on the approach to exacerbation diagnosis and treatment.

For the first time, this study aimed to determine the association of URT symptoms with HRV prevalence and load, in the stable state COPD, at exacerbation and during the recovery period, in naturally occurring exacerbations.

5.2 Characteristics of patients used in stable state and exacerbation analysis

A total of 58 stable state samples and 272 exacerbation samples (7 of the 279 exacerbation samples had missing URT symptom data) from 146 COPD patients were analysed between January 2008 and March 2014 as part of the sample collection performed in the London COPD cohort. The baseline clinical characteristics of the 146 patients included are shown in Table 5.2 below.

FEV ₁ , litres	1.18 (\pm 0.5)
FVC, litres	2.56 (\pm 0.9)
FEV ₁ /FVC, %	46.1 (\pm 14.8)
Predicted FEV ₁ , %	47.0 (\pm 21.7)
Current Smoker, %	33
Age, years	69.8 (\pm 8.5)
Male gender, %	59

Table 5.2: Baseline clinical characteristics of 146 COPD patients in the London COPD Cohort, who participated in study of HRV infection and upper respiratory tract symptoms at COPD exacerbation (n=272) and in the stable state (n=58). Data are presented as mean (\pm SD) or percentage (%).

5.3 Results using stable state and exacerbation samples

5.3.1 URT symptom prevalence in different disease states

In the stable state, cold symptoms were reported in 16 of the 58 samples (27.6%). This prevalence of cold symptoms increased significantly at exacerbation where 115 of the 272 samples (42.3%) had reported cold symptoms; $p=0.038$ (Figure 5.2).

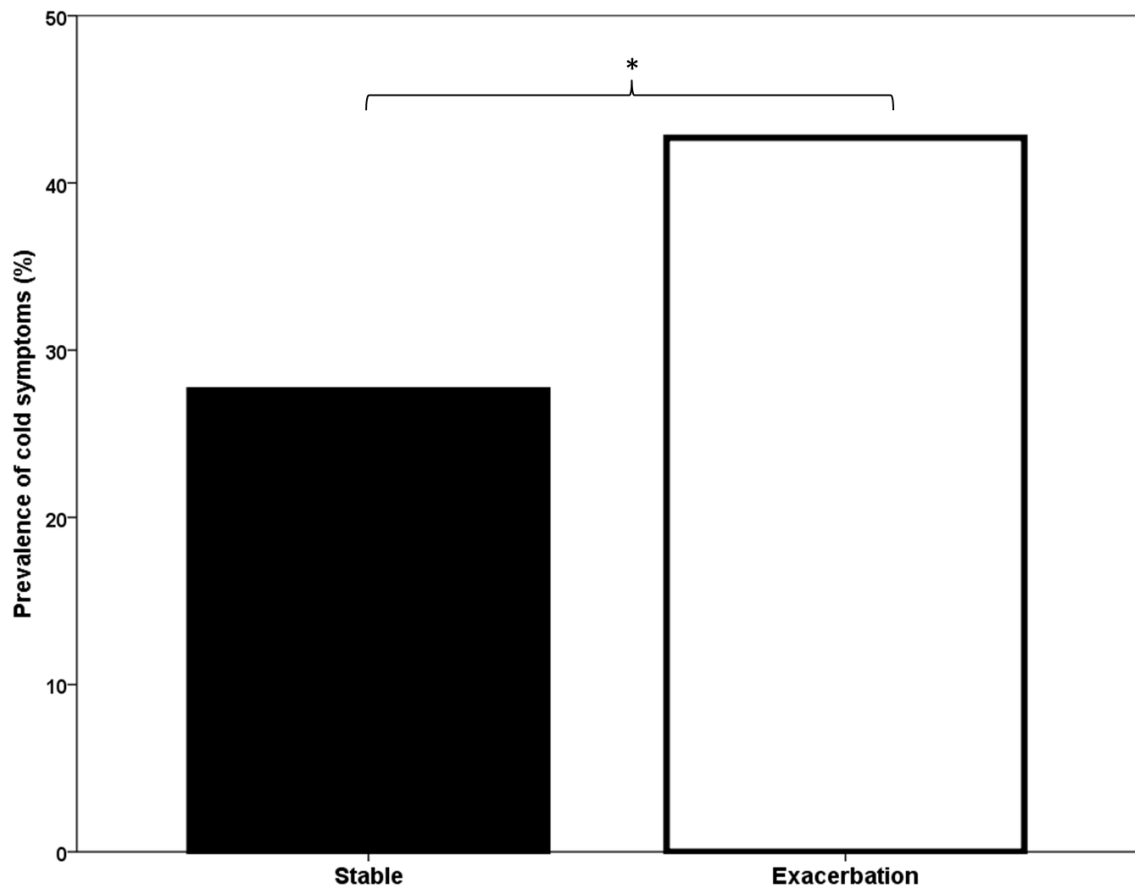


Figure 5.2: Comparison of the prevalence of cold symptoms in stable (n=58) and exacerbation (n=272) samples; * $p=0.038$

Sore throats were reported in 2 of the 58 stable samples (3.4%), but increased significantly at exacerbation where 38 of the 272 samples (14%) had reported sore throats; $p=0.026$ (Figure 5.3).

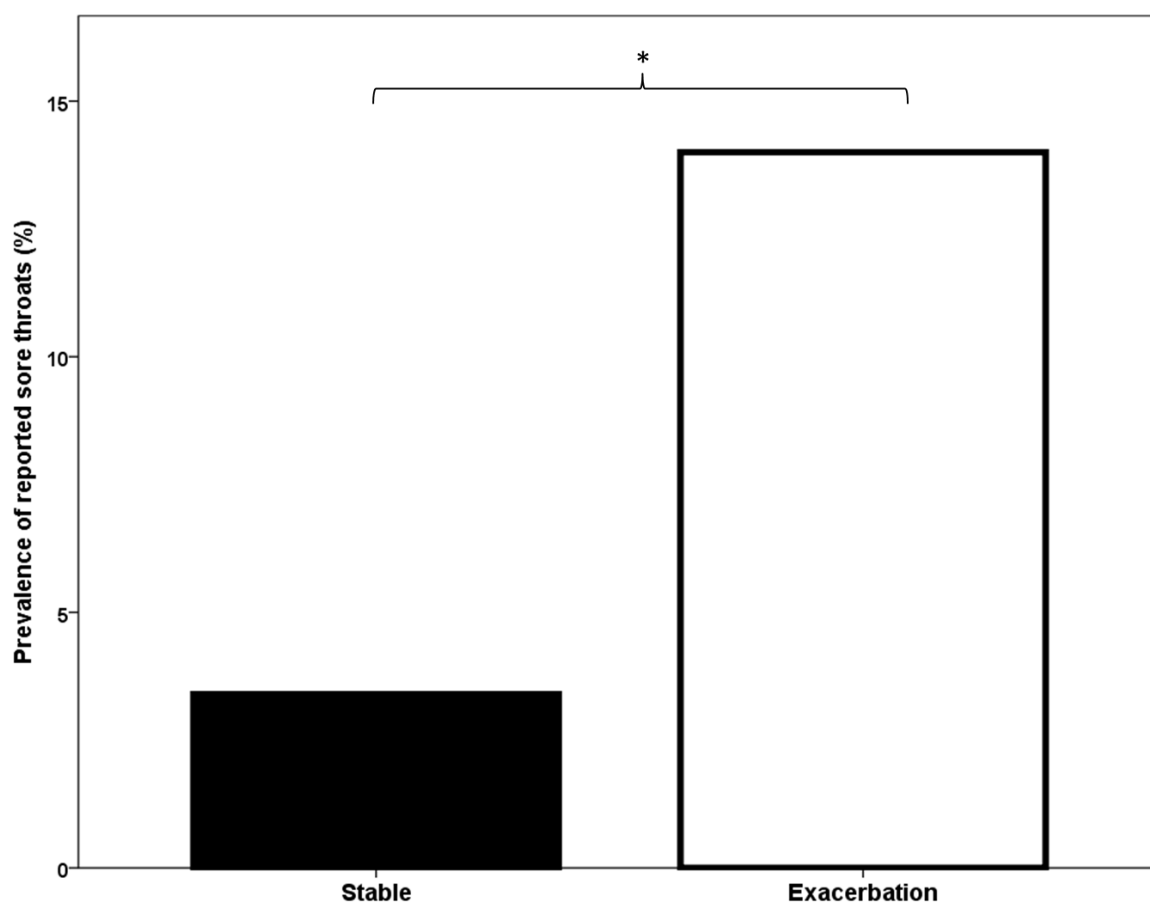


Figure 5.3: Comparison of the prevalence of sore throats in stable (n=58) and exacerbation (n=272) samples; * $p=0.026$

5.3.2 HRV prevalence with URT symptoms

At exacerbation (n=272), 115 samples were associated with cold symptoms and 157 were not. Of the 115 samples that were associated with cold symptoms, 73 (63.5%) were found to positive for HRV. This was significantly higher than in the 157 samples not associated with cold symptoms where 44 (28%) were positive for HRV; $p<0.001$ (Figure 5.4).

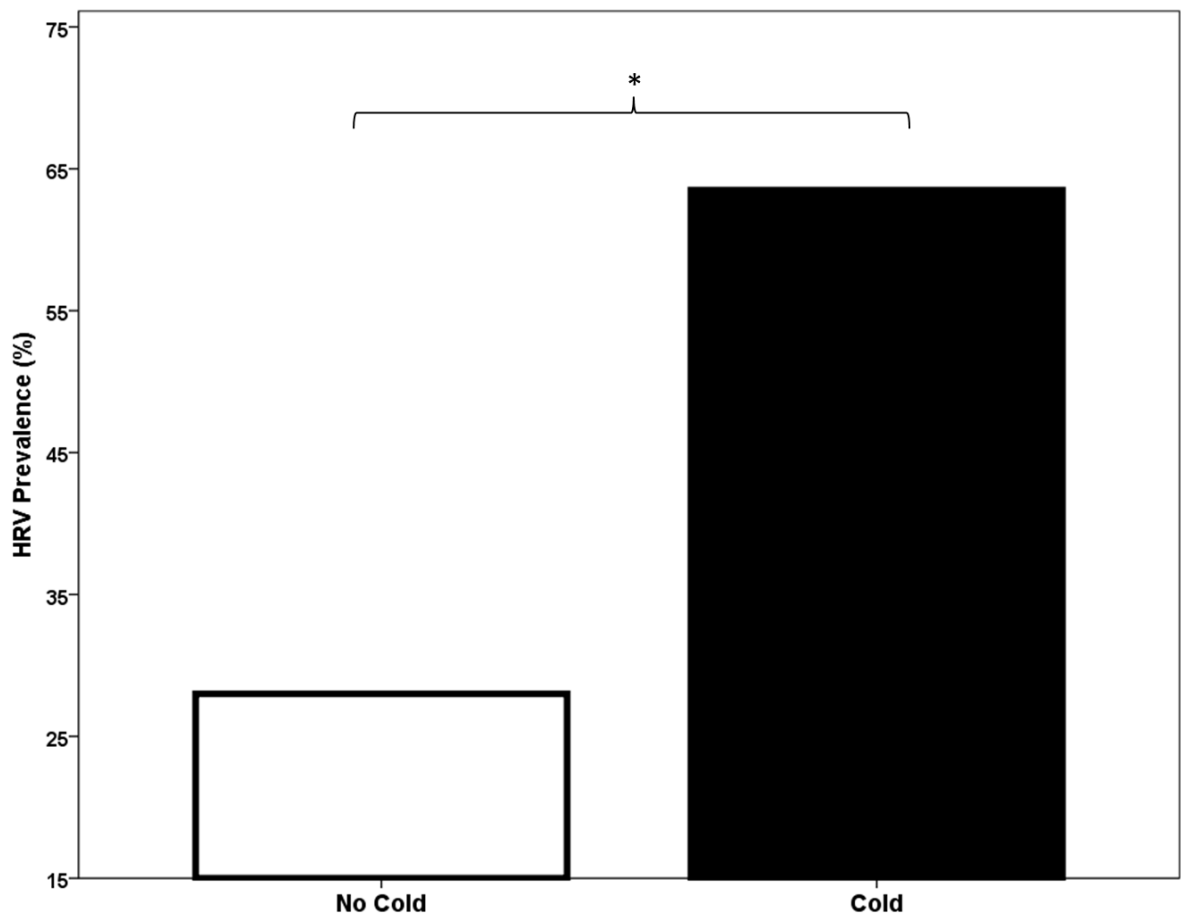


Figure 5.4: Comparison of the prevalence of HRV infection in samples with cold symptoms (n=115) and samples no cold symptoms (n=157), at exacerbation; * $p<0.001$.

Of the same 272 exacerbation samples, 38 samples were associated with sore throats and 234 were not associated. In the 38 samples that were associated with sore throats, 29 (63.5%) were found to be positive for HRV which was significantly higher than in the 234 samples not associated with cold symptoms where 92 (39.3%) were positive for HRV infection; $p=0.002$ (Figure 5.5).

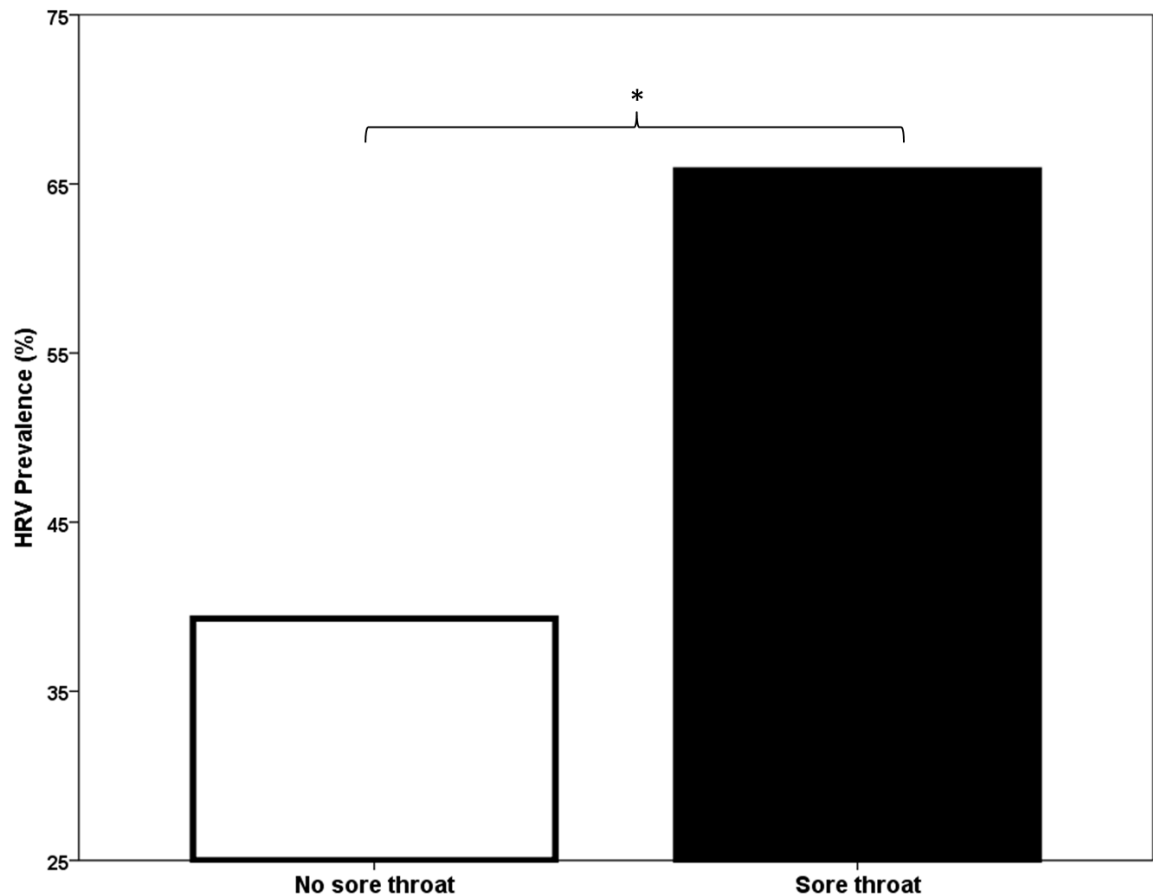


Figure 5.5: Comparison of the prevalence of HRV infection in samples with sore throats (n=38) and samples without sore throats (n=234), at exacerbation; * $p=0.002$.

5.3.3 URT symptom prevalence with HRV

The prevalence of cold symptoms at exacerbation was significantly higher in those with (n=117) HRV infection (62.4%) compared to those without (n=155) HRV infection (27.1%); $p<0.001$ (Figure 5.6).

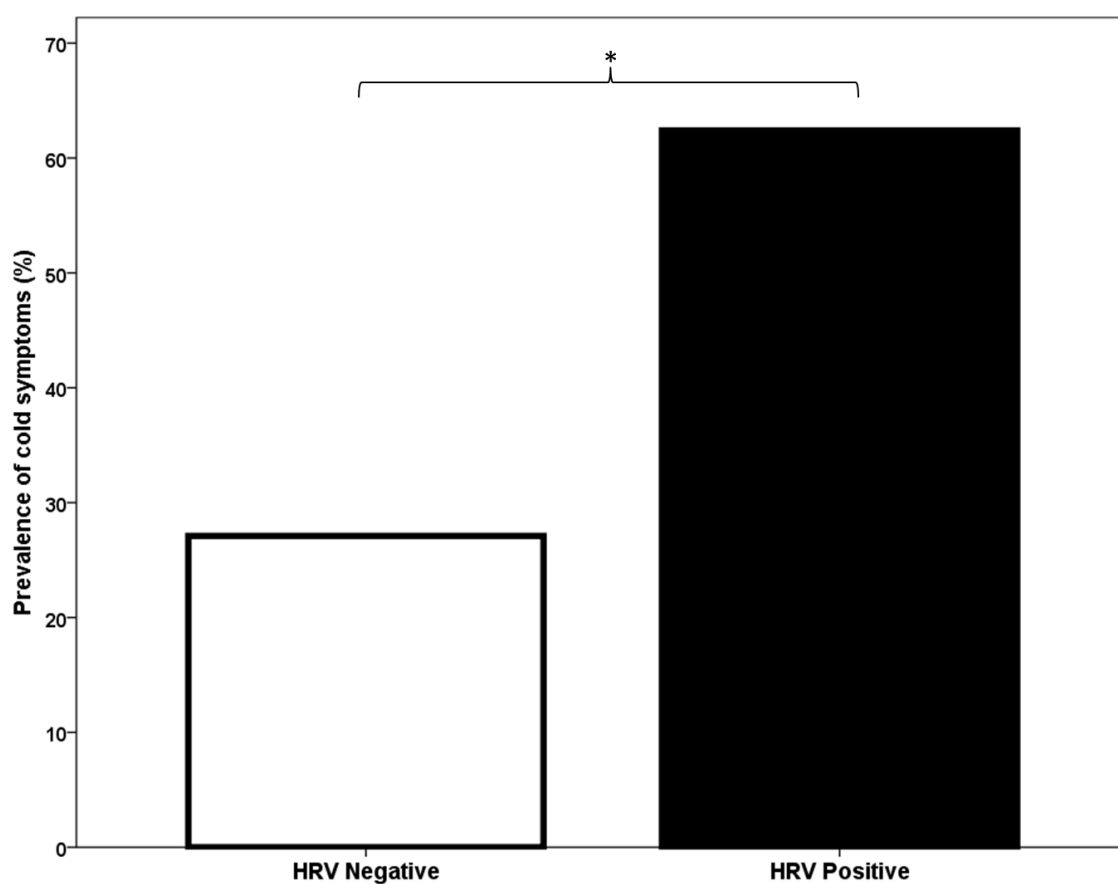


Figure 5.6: Comparison of the prevalence of cold symptoms in samples positive for HRV (n=117) and negative for HRV (n=155), at exacerbation; * $p<0.001$.

The prevalence of sore throats was significantly higher in those with (n=117) HRV infection (21.4%) compared to those without (n=155) HRV infection (8%); $p=0.002$ (Figure 5.7).

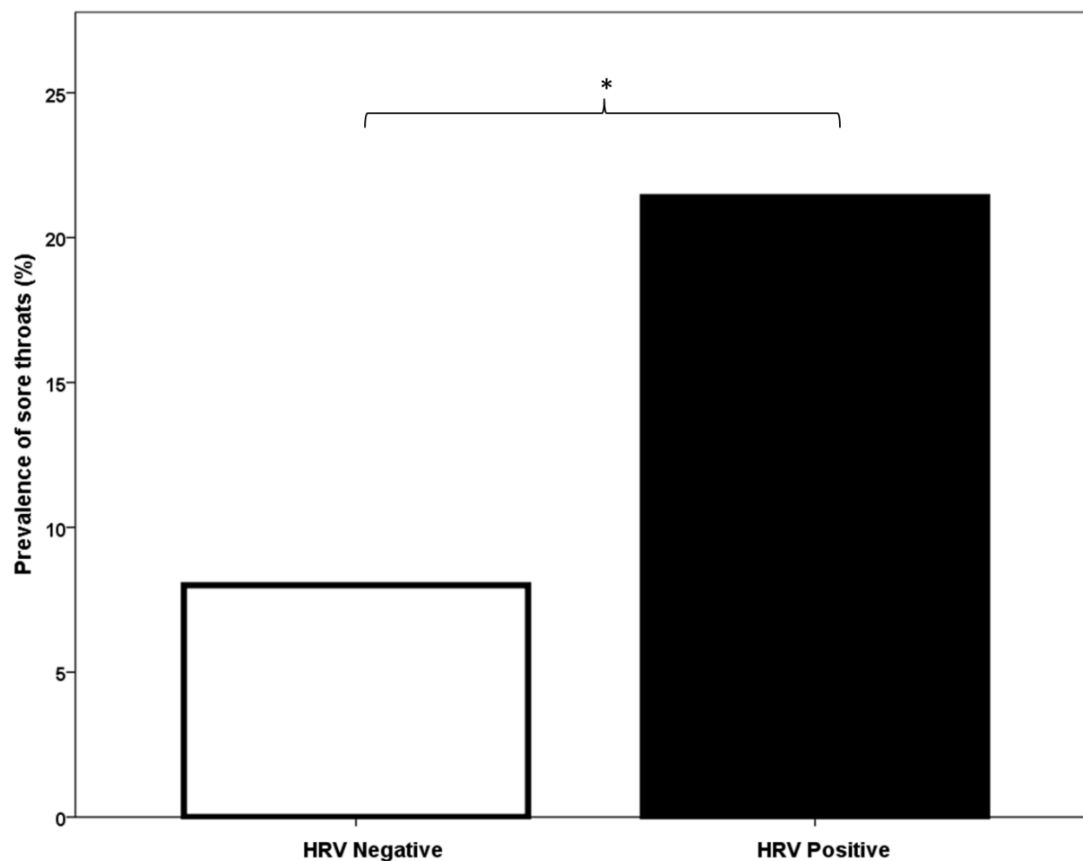


Figure 5.7: Comparison of the prevalence of sore throats in samples positive for HRV (n=117) and negative for HRV (n=155), at exacerbation; * $p<0.001$.

5.3.4 HRV load with URT symptoms

HRV-positive exacerbation samples associated with cold symptoms (n=73) had a median (IQR) HRV load of $10^{4.49(2.49-5.50)}$ pfu/ml which was significantly higher than in HRV-positive exacerbations without cold symptoms (n=44), $10^{2.51(1.62-4.59)}$ pfu/ml; p=0.004 (Figure 5.8).

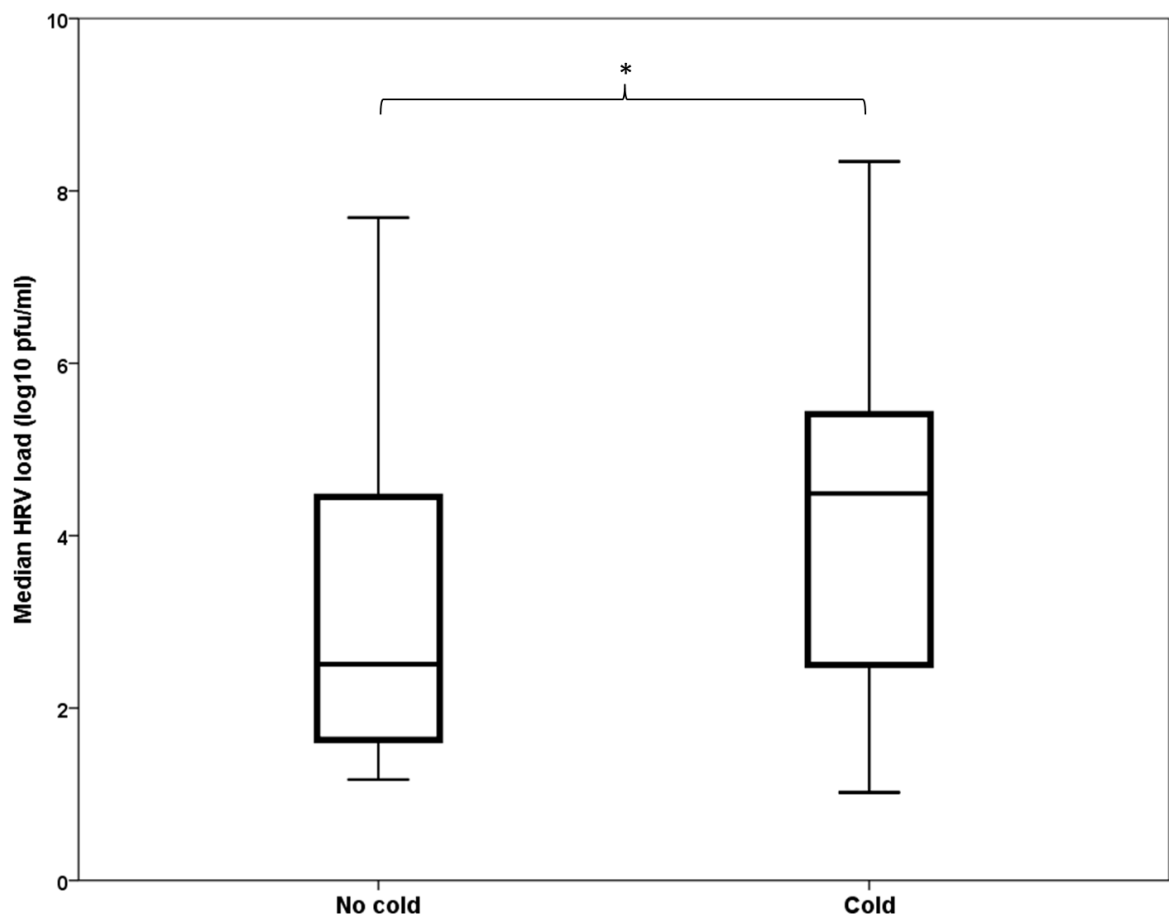


Figure 5.8: Comparison of HRV load in HRV-positive samples with cold symptoms (n=73) and HRV-positive samples without cold symptoms (n=44), at exacerbation; *p=0.004. Data presented as median (IQR).

The median (IQR) HRV load was significantly higher when a sore throat was reported (n=25), $10^{4.91(3.76-6.56)}$ pfu/ml compared to samples without a reported sore throat (n=92), $10^{3.06(1.66-4.87)}$ pfu/ml; $p<0.001$ (Figure 5.9).

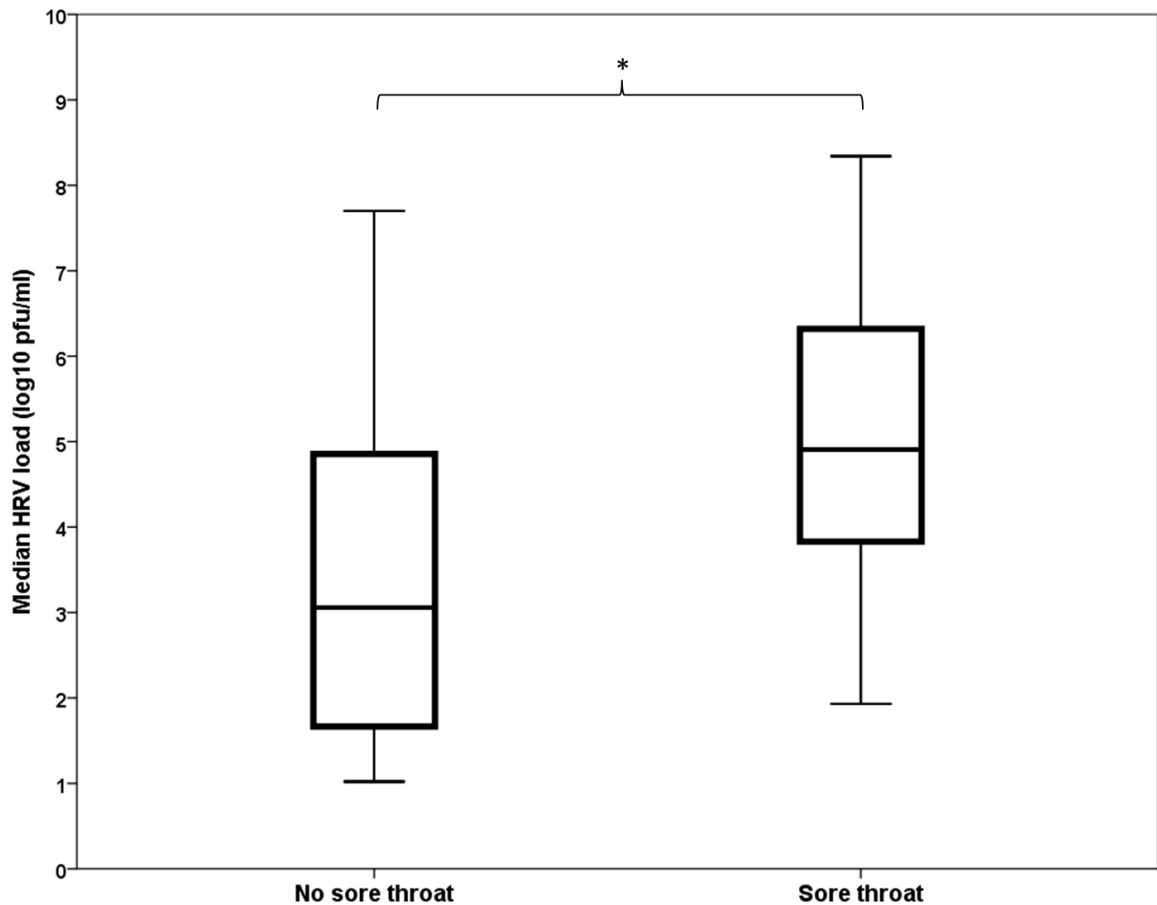


Figure 5.9: Comparison of HRV load in HRV-positive samples with sore throats (n=25) and HRV-positive samples without sore throats (n=92), at exacerbation; * $p<0.001$.

When looking at the median (IQR) HRV load in exacerbation samples associated with cold symptoms only (n=58), sore throats only (n=8) or both symptoms (n=17), the load was

significantly higher in those that had both symptoms ($10^{6.13(4.07-7.18)}$ pfu/ml) compared to those that had cold symptoms only ($10^{3.84(1.81-5.16)}$ pfu/ml; $p=0.002$) or a sore throat only ($10^{4.00(2.56-5.12)}$ pfu/ml; $p=0.049$) (Figure 5.10).

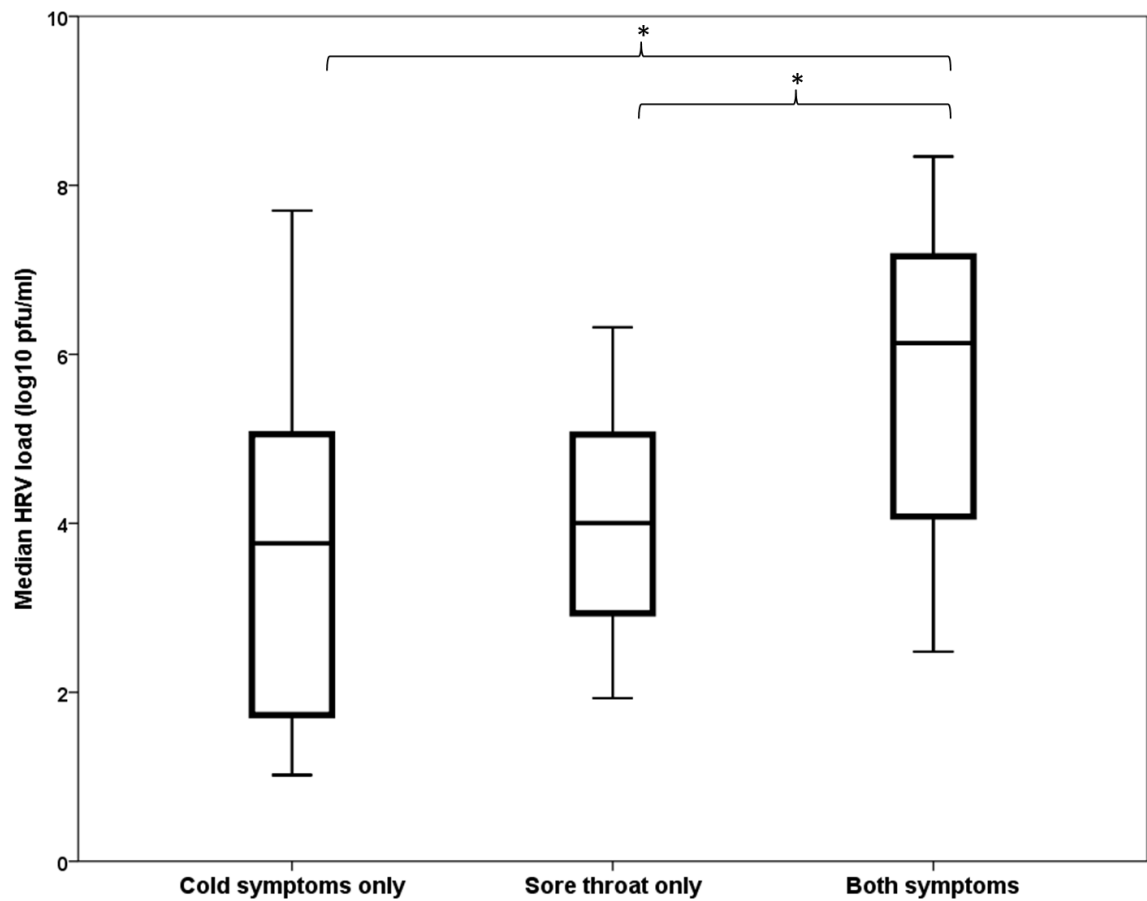


Figure 5.10: Comparison of the HRV load at exacerbation in samples associated with cold symptoms only (n=56), sore throats only (n=8), or both symptoms (n=17); * $p<0.05$. Data shown as median (IQR).

The number of HRV-negative exacerbations (n=155) that had cold symptoms was 42 (27.1%), 13 (8.4%) had a sore throat and 4 (2.6%) had both symptoms.

5.4 Characteristics of patients used in time-course analysis

A total of 537 samples; 129 taken at exacerbation presentation, 92 at Day 3, 115 at Day 7, 102 at Day 14, 77 at Day 35 and 22 at Day 56 samples from 71 COPD patients were obtained between April 2010 and June 2013. The baseline clinical characteristics for these patients are shown in Table 5.3.

FEV ₁ , litres	1.19 (\pm 0.5)
FVC, litres	2.45 (\pm 0.8)
FEV ₁ /FVC, %	46.2 (\pm 14.9)
Predicted FEV ₁ , %	45.9 (\pm 20.3)
Current Smoker, %	33
Age, years	72 (\pm 8.0)
Male gender, %	61

Table 5.3: Baseline clinical characteristics of 71 patients in the London COPD Cohort who participated in study of HRV infection with upper respiratory tract symptoms during COPD exacerbation and recovery. Data are presented as mean (\pm SD) or percentage (%).

5.5 Results using time-course sample analysis

5.5.1 URT symptom prevalence during the time-course

At exacerbation presentation (ExP), the percentage of samples that had associated cold symptoms was found to be 52.7% (68/129). This prevalence decreased significantly during the recovery period to 34.8% at Day 7, 20.6% at Day 14, 15.6% at Day 35 and 22.7% at Day 56 (all $p < 0.009$) (Figure 5.11).

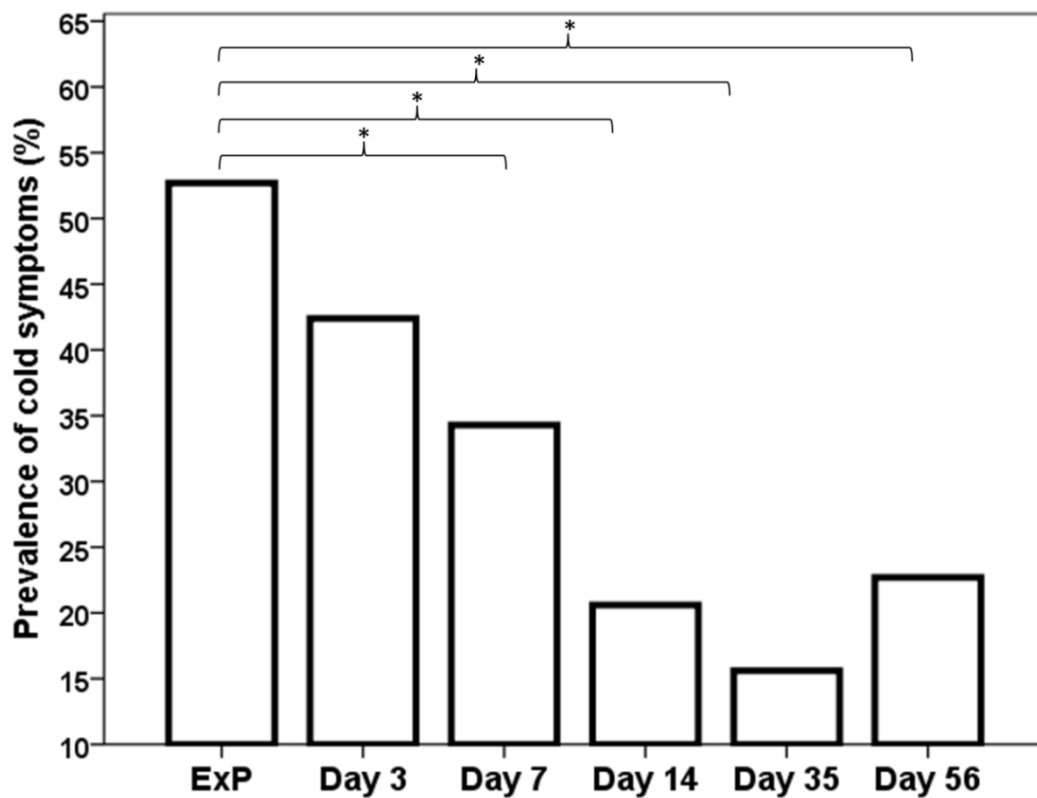


Figure 5.11: Change in the prevalence of cold symptoms during the time-course of an exacerbation and recovery period; * $p < 0.01$.

At exacerbation presentation (ExP), the percentage of samples that had associated sore throat was found to be 23.3% (30/129). This prevalence decreased significantly during the time-course of the recovery period to 9.8% at Day 14 and 3.9% at Day 35 (both $p < 0.007$) (Figure 5.12).

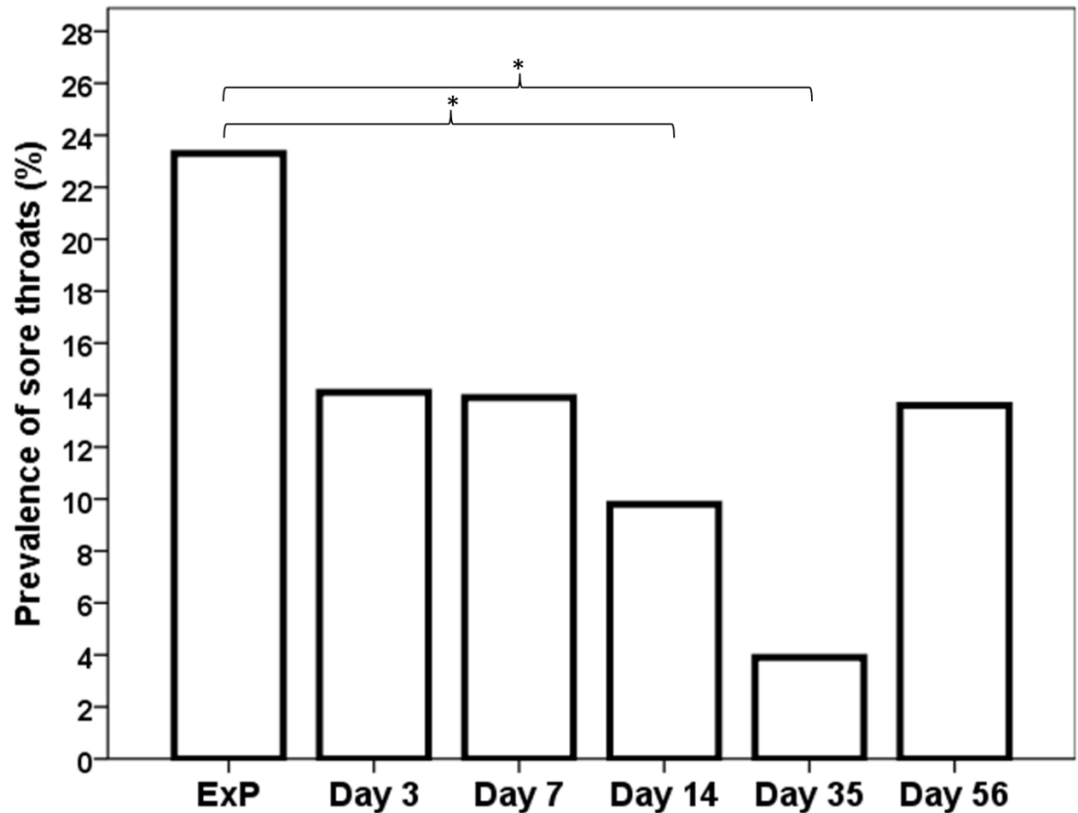


Figure 5.12: Change in the prevalence of sore throats during the time-course of an exacerbation and recovery period; * $p < 0.01$.

5.5.2 HRV prevalence with URT symptoms over the time-course

The absolute prevalence values of HRV were found to be higher at exacerbation presentation (ExP) and at each time point during the recovery period until Day 35, in samples associated with cold symptoms compared to those not associated, (Figure 5.13) however this missed statistical significance for each time point (all $p \geq 0.132$) except at Day 7. At Day 7, the HRV prevalence in samples associated with cold symptoms (37.5%) was significantly higher than in samples not associated with cold symptoms (16%) ($p=0.016$).

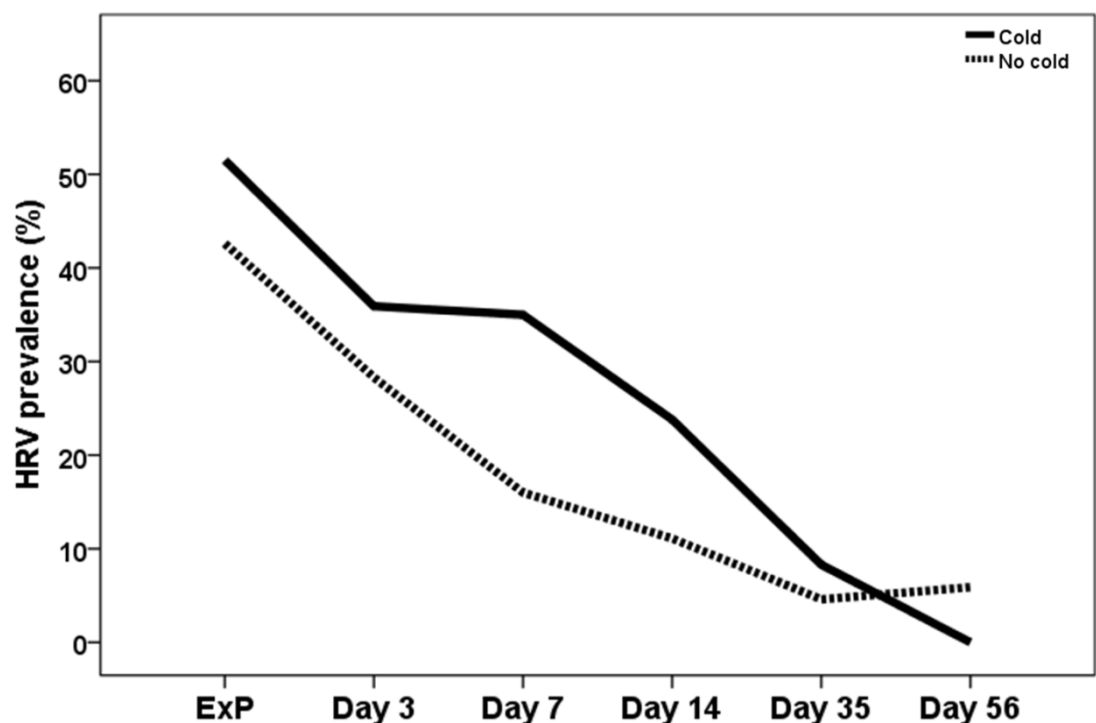


Figure 5.13: Comparison of the changes in the prevalence of HRV over the time-course of an exacerbation and recovery in samples associated with cold symptoms and those without cold symptoms.

The absolute prevalence values of HRV were found to be higher at ExP and at each time point during the recovery period until Day 35, in samples associated with sore throats compared to those not associated (Figure 5.14), however this did not reach statistical significance at any time point ($p \geq 0.115$) except Day 3. At Day 3 the HRV prevalence in samples associated with sore throats (53.8%) was significantly higher than in samples not associated with sore throats (25.3%) ($p=0.036$).

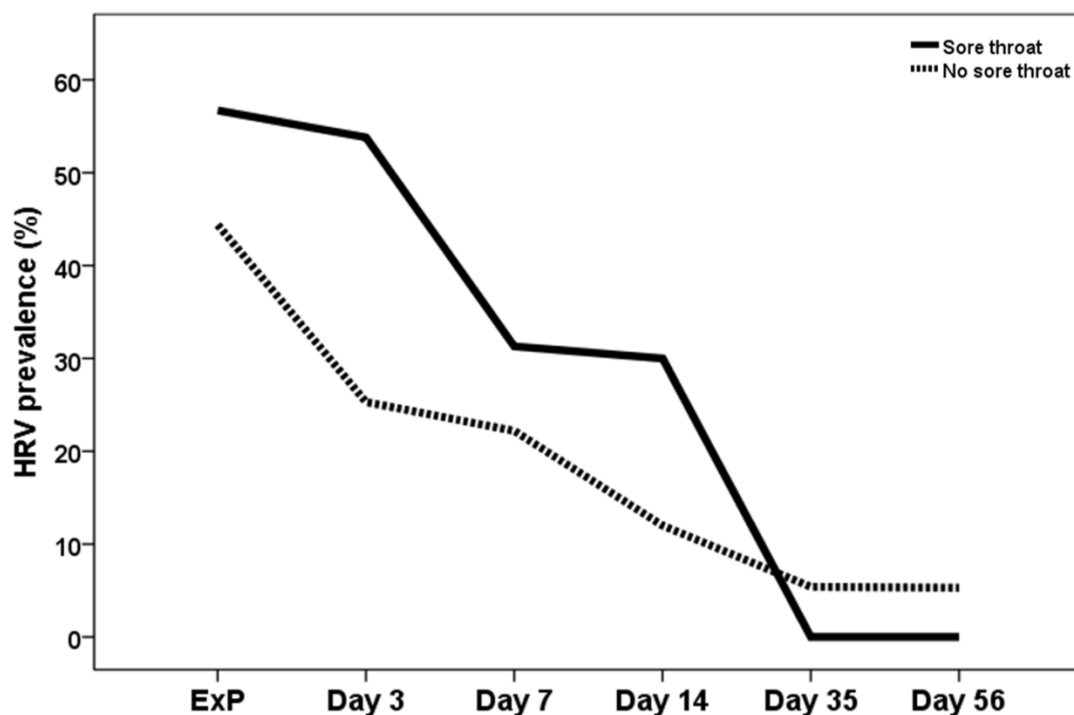


Figure 5.14: Comparison of the changes in the prevalence of HRV over the time-course of an exacerbation and recovery in samples associated with sore throats and those without sore throats.

The prevalence of HRV was found to be significantly higher in samples associated with both cold symptoms and sore throats (57.1%) compared to the HRV prevalence in samples with either cold symptoms only (29.9%) or sore throats only (27.3%) in samples from all time points; both $p < 0.010$ (Figure 5.15).

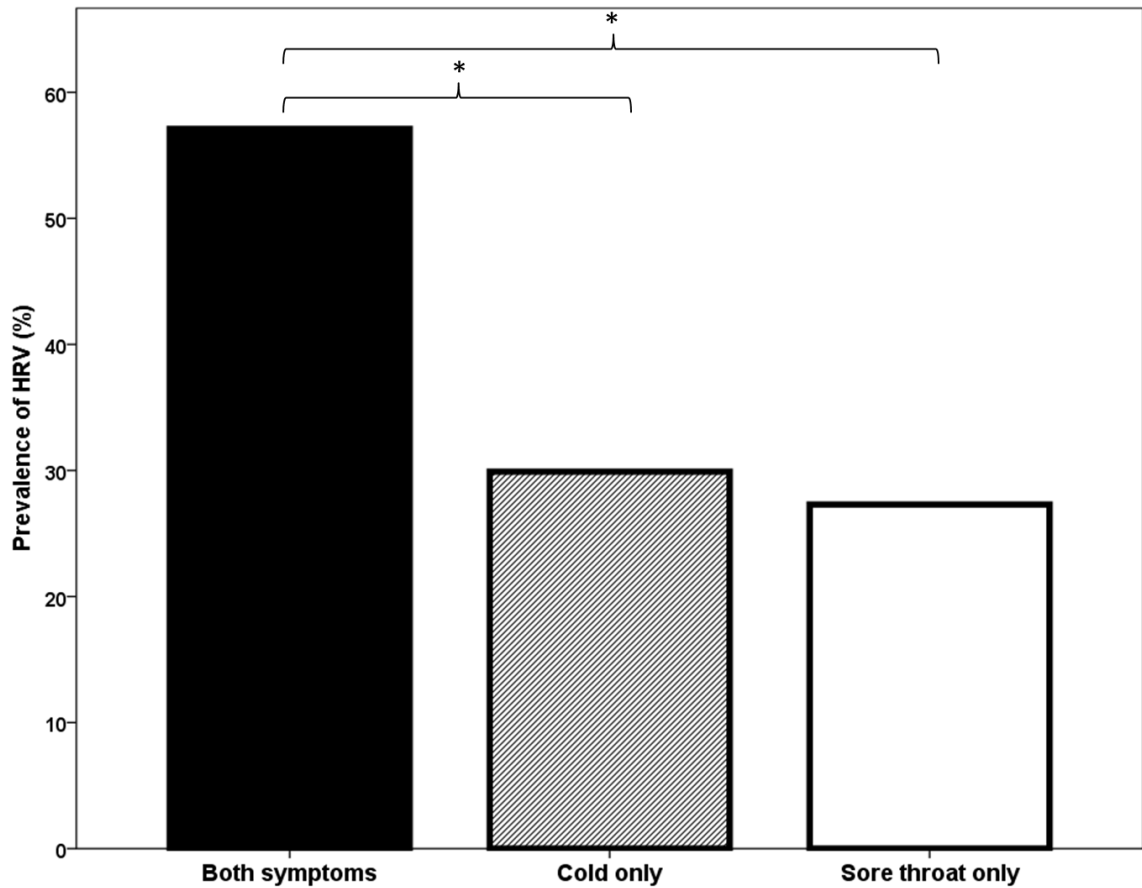


Figure 5.15: Differences in the prevalence of HRV in samples associated with both cold symptoms and sore throats and those with cold symptoms only or sore throats only, using all time points; * $p < 0.01$.

5.5.3 HRV load with URT symptoms over the time-course

In exacerbations positive for HRV at presentation (ExP), the median (IQR) HRV load was significantly higher in patients who reported cold symptoms at ExP (n=34) compared to those that did not (n=26), $10^{4.29(2.25-5.22)}$ pfu/ml vs $10^{2.35(1.39-4.09)}$ pfu/ml respectively; $p=0.006$. The median HRV load was also significantly higher in patients who reported cold symptoms compared to those that did not at Day 3 ($10^{1.91(0-4.16)}$ pfu/ml vs $10^{0(0-1.77)}$ pfu/ml respectively; $p=0.046$), and at Day 7, ($10^{0(0-3.17)}$ pfu/ml vs $10^{0(0-0)}$ pfu/ml respectively; $p=0.029$).

In both groups, the HRV load fell over the recovery period being highest at Day ExP and zero at Day 56. There was a significant fall in the median HRV load from Day ExP to Day 3. In patients who reported cold symptoms the HRV load fell from $10^{4.29(2.25-5.22)}$ pfu/ml at ExP to $10^{1.91(0-4.16)}$ pfu/ml at Day 3 ($p=0.006$) and in patients that did not report cold symptoms the load fell from $10^{2.35(1.39-4.09)}$ pfu/ml at ExP to $10^{0(0-1.77)}$ pfu/ml at Day 3 ($p<0.001$). There was no significant change between any other time-points during the time-course in either group (Figure 5.16).

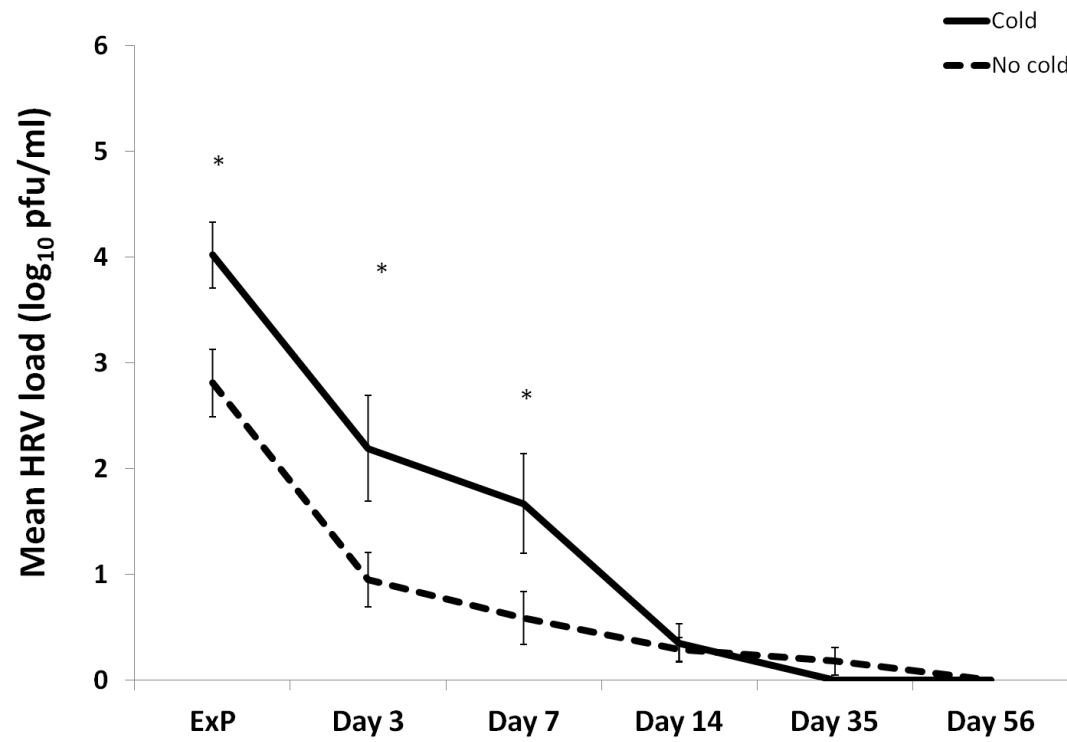


Figure 5.16: The change in HRV load over the time-course of an exacerbation and recovery period in samples with cold symptoms and those without cold symptoms. Data shown as mean (\pm SEM).

In patients who reported a sore throat, the median HRV load was significantly higher at ExP and Day 3 compared to those without a sore throat (both $p \leq 0.018$). In those that reported a sore throat, the HRV load at ExP was $10^{4.91(3.92-5.60)}$ pfu/ml compared to $10^{2.61(1.64-4.61)}$ pfu/ml in those that did not ($p=0.006$). At Day 3, the HRV load was $10^{4.14(1.77-4.82)}$ pfu/ml in patients who reported a sore throat compared to $10^{0(0-1.82)}$ pfu/ml in those that did not ($p=0.002$) (Figure 5.17).

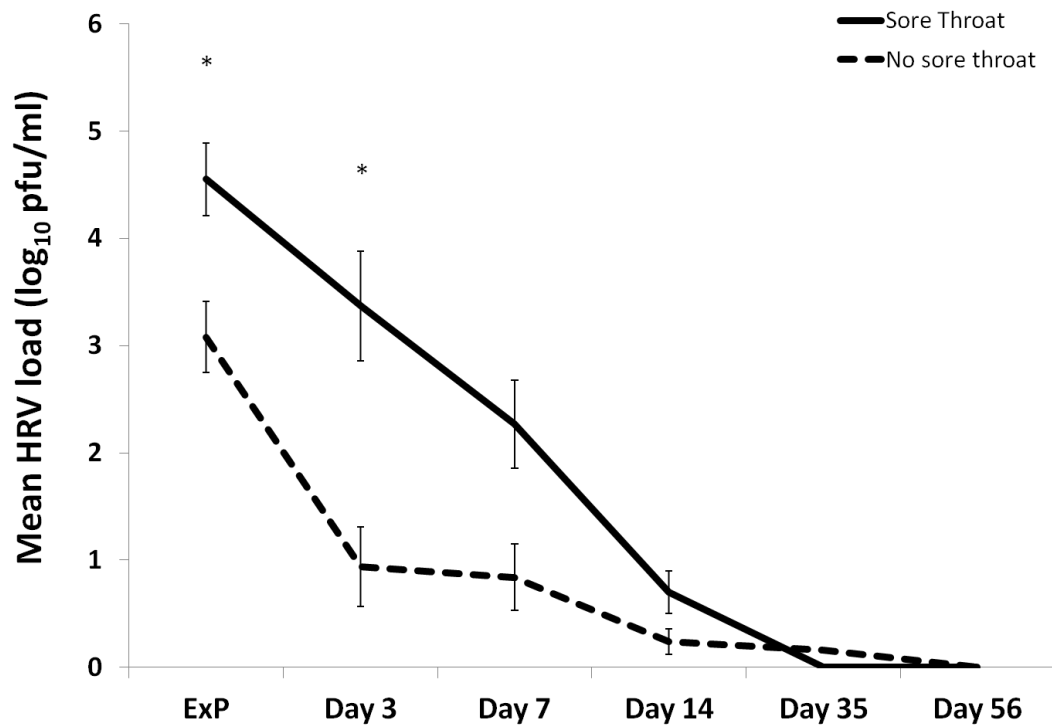


Figure 5.17: The change in HRV load over the time-course of an exacerbation and recovery period in samples associated with a sore throat and those without a sore throat. Data shown as mean (\pm SEM).

The median HRV load was significantly higher in patients who had both cold symptoms and a sore throat compared to those that had either cold symptoms only or a sore throat only, at Day 7 post-exacerbation; the median (IQR) HRV load was $10^{4.48(1.85-7.28)}$ pfu/ml in those with both symptoms (n=4) compared to $10^{0(0-2.74)}$ pfu/ml in those with cold symptoms only (n=19) (p=0.018) and $10^{0(0-0)}$ pfu/ml in those with a sore throat only (n=4) (p=0.029). At Day 3, the median (IQR) HRV load in samples associated with both symptoms (n=5) was $10^{4.22(3.94-4.88)}$ pfu/ml which was significantly higher than in those that had cold symptoms only (n=9) ($10^{0.55(0-1.98)}$; p=0.002) but not in those with a sore throat only (n=2) ($10^{0.89(0-0.89)}$; p=0.095). The median HRV load decreased significantly from ExP to Day 3 in samples associated with either cold symptoms (p<0.001) or a sore throat (p=0.049). In those associated with both symptoms however, there was no decrease in median HRV load between these time-points (Figure 5.18).

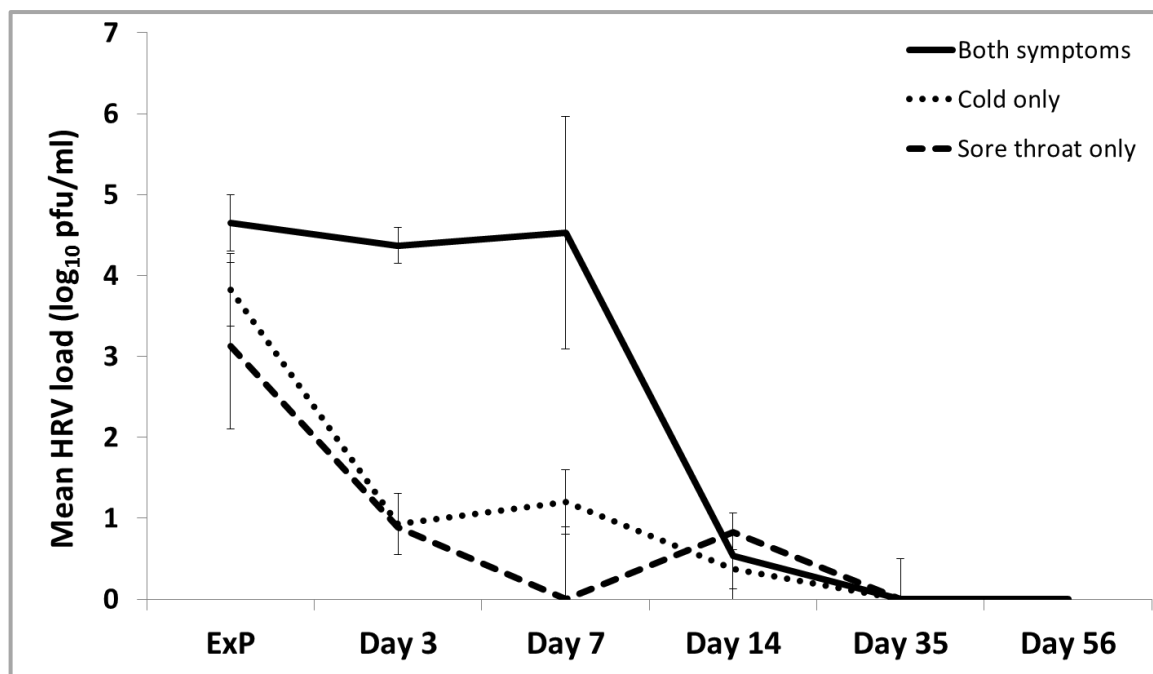


Figure 5.18: The change in HRV load over the time-course of an exacerbation and recovery period in samples associated with both cold symptoms and a sore throat (n=23), cold symptoms only (n=67) or a sore throat only (n=16). Data shown as mean (\pm SEM).

5.6 Discussion

This study investigated the relationship between HRV infection and upper respiratory tract (URT) symptoms, namely cold symptoms and sore throats, in the stable state, at exacerbation presentation and during recovery until Day 56 post-exacerbation, in natural exacerbations of COPD. It also explored changes in URT symptoms in patients who were positive for HRV infection and those that were not, and studied these changes in relation to the load of HRV as well as the prevalence. For the first time, the association of HRV infection and URT symptoms during the complete time-course of naturally occurring COPD exacerbations has been investigated.

A study in 2003 which investigated symptom changes at different COPD disease states, showed an increase in URT symptoms from the stable state to COPD exacerbation, where cold symptoms increased from 23.8% in the stable state to 38.8% at exacerbation ($p=0.040$), and sore throats increased from 2.4% to 22.4% ($p=0.01$) (Rohde et al., 2003). In 2000, Seemungal and colleagues found 61% of exacerbations were associated with colds and 30% with sore throats (Seemungal et al., 2000). The current data supports these findings and has found that the prevalence of both cold symptoms and sore throats increased significantly from the stable state to exacerbation, verifying a relationship between an increase in URT symptoms and the onset of COPD exacerbations.

URT symptoms have been shown to be particularly associated with virus-associated exacerbations. Kherad and colleagues found cold symptoms in 45% of samples negative for HRV but 73% of HRV-positive samples using nasopharyngeal samples (Kherad et al., 2010) and Seemungal and colleagues showed that viral-associated exacerbations had a significantly greater daily symptom score (median=3) compared to non-viral-associated exacerbations (median=2) ($p=0.009$) (Seemungal et al., 2001). The current study has shown that in the presence of HRV, the prevalence of URT symptoms was significantly higher than when HRV infection was not present. At exacerbation, the prevalence of HRV was significantly higher in patients who reported URT symptoms compared to those that did not. These findings suggest that URT symptoms may indicate the presence of an HRV infection which could have significant implications in how these exacerbations are treated clinically. Currently, there are no antiviral agents in use for the treatment of HRV infections (Mallia et al., 2007). Unlike antivirals such as amantadine and zanamivir which have shown excellent activity against influenza viruses (McKinlay, 2001), most of the investigations into the development of antivirals against HRV have failed to show benefits in human clinical trials due to adverse events, intolerance and limited potency (McKinlay, 2001). Pleconaril is an anti-viral drug that was developed in 1997 for the prevention of asthma exacerbations and common cold symptoms in patients exposed to HRV infections (Turner and Hendley, 2005). It works by inhibiting *in vitro* replication of most rhinoviruses and enteroviruses. It was shown to reduce cold symptom scores and reduce the frequency of picornavirus infection cultured from nasal mucus specimens. However it was also

associated with a higher incidence of nausea and diarrhoea compared to placebo (Hayden et al., 2003) and this antiviral is no longer in use. The development of a vaccine for HRV is proving difficult due to reasons such as there being over 100 serotypes of HRV with high sequence variability in their antigenic sites (Palmenberg et al., 2009). Furthermore, unlike influenza, there is limited knowledge of HRV epidemiology as HRV surveillances are restricted, and also, there are limited animal models for HRV infection and evidence of HRV pathophysiology is still scarce (Rohde, 2011). Despite this, and despite not yet being close to the clinical development of an HRV vaccine, due to the large number of HRV serotypes (Glanville et al., 2013), current research including a study by Edlmayr and colleagues, that showed strong cross-neutralising activity for different HRV strains (Edlmayr et al., 2011), the development of an HRV vaccine does seem feasible. It must be considered that infection with other viruses may lead to the onset of cold symptoms and sore throats as well as HRV (Seemungal et al., 2001) and that these viruses may also play a role in URT symptom development. However the findings from the current study show an association between URT symptoms and HRV infection which must be addressed in terms of specific therapy for HRV-associated exacerbations.

To date, the association of URT symptoms and HRV infection over the entire time-course of an exacerbation and recovery period has not been investigated in naturally occurring exacerbations of COPD. As discussed previously, the study by Mallia in 2011 in which patients with mild COPD and healthy controls were experimentally infected with low-dose

HRV showed a significant increase in URT symptoms at 2-14 days post-inoculation with HRV with symptom counts decreasing during recovery (Mallia et al., 2011). Additionally, the study of experimental HRV infection in non-smoking asthmatics found that in patients experimentally infected with HRV, cold symptoms started to increase from Day 0, peaking on Day 2 before decreasing during exacerbation recovery which was significantly different from the placebo group (Grunberg et al., 1999). The current study, for the first time, explored HRV infection over the time-course of exacerbation and recovery in relation to URT symptoms in natural exacerbations and reported similar findings to the experimental studies described above. The prevalence of URT symptoms was highest at exacerbation onset and decreased during recovery up until Day 35 post-exacerbation. The current study has shown that at exacerbation presentation and each time point during recovery until Day 35, patients who reported having URT symptoms had a higher absolute prevalence of HRV compared to those that did not. HRV load was significantly higher in patients reporting cold symptoms or a sore throat at exacerbation presentation, Day 3 and Day 7 post-exacerbation compared to patients without those symptoms. This is an important area of study as measuring the pattern of URT symptoms, not only at exacerbation, but during recovery, may give important insight into the pattern of HRV infection which in turn may impact on the treatment of exacerbations. Treatment with antiviral or anti-inflammatory therapy at this point would be preferable. However, as discussed previously, there are no current antivirals for the specific treatment of HRV infection in use at present. Therefore, the development of guidelines with the aim of assisting clinicians in

the treatment of the sub-population of HRV exacerbating COPD patients may be the best approach now there is an effective method of early identification of HRV in these patients. Furthermore, these results may aid clinicians in counselling patients with URT symptoms about their exacerbation potentially lasting longer than other non-viral associated exacerbations, and that they do not need to seek antibacterial medication at that time.

Not only was the HRV prevalence and load higher in the presence of URT symptoms compared to without symptoms, the prevalence of HRV was significantly higher at exacerbation in patients who reported both cold symptoms and a sore throat compared to those who reported just one of the symptoms alone. Data from this study showed that in patients who reported having either cold symptoms or a sore throat, the HRV load decreased significantly by Day 7. However in patients who reported both symptoms at the time of sampling, the HRV load remained elevated until Day 14. This suggests patients who have more symptoms at exacerbation have higher HRV loads than those with less symptoms, which may indicate more severe exacerbations in terms of greater burden of symptoms, and that exacerbations with both symptoms have longer recovery times compared to those with only one symptom. Seemungal and colleagues showed in 2001 that patients positive with viral infection had a significantly longer recovery time in terms of more symptoms than non-viral associated exacerbations (Seemungal et al., 2001). Whether having a higher viral load means a more severe exacerbation compared to those with lower loads, has not been explored to date, however the current data shows that

patients with higher HRV loads have more symptoms, and therefore potentially more severe exacerbations due to these symptoms. The current findings also support the work by Seemungal and colleagues, in that in patients with more symptoms, the HRV load remained higher for a longer period of time than in patients with only one symptom, suggesting longer recovery times for more symptomatic patients.

URT symptoms have been shown to begin before the onset of an exacerbation. In 2000 it was shown by Seemungal and colleagues that cold symptoms precede an exacerbation with symptoms scores increasing during the prodromal period (7 days before the onset of the exacerbation) (Seemungal et al., 2000). In the current study, the median number of days between the onset of cold symptoms and exacerbation presentation to the clinic was just 2 days, and just 1 day between the onset of a sore throat and exacerbation presentation. Findings from both the Seemungal study and the current study suggest that URT symptoms could be a potential indicator of viral infection in these patients. Conversely, this short time frame between symptom onset and exacerbation presentation demonstrates how patients are sampled early in their exacerbation in the London COPD cohort. A unique advantage of the London COPD Cohort is that patients are prospectively followed and reviewed regularly, allowing the identification of exacerbation symptoms early at exacerbation onset, therefore optimising viral detection and enabling study of the time-course of these events. This allows patients to be treated early in their exacerbation, ideally leading to rapid improvement of their symptoms and therefore a less severe, or

shorter, exacerbation. Late sampling of an exacerbation, that is when the exacerbation is underway and not at the onset, can lead to missed viral detection and therefore poor detection rates. It was shown in Chapter 4 that it is difficult to find an accurate and reliable marker of HRV infection in COPD patients, which stresses the importance of these findings all the more, as URT symptoms could be used as a potential marker of HRV infection.

The results of the current study confirm the association of upper airway symptoms and HRV infection, and importantly, emphasise that the presence of HRV may be indicated by the presence of cold symptoms and/or sore throats. These findings may impact on HRV-associated exacerbation treatment strategies now there is an effective method of identifying HRV infections in COPD patients.

5.7 Conclusion

For the first time, this study has shown a relationship between HRV infection and upper airway symptoms during the entire time-course of naturally occurring exacerbations and recovery period. It has been shown throughout the time-course that patients with URT symptoms have significantly higher HRV loads than those without, which reinforces the concept of URT symptoms and HRV associations. This study highlighted the importance of early sampling of COPD patients at exacerbation in order to study their pathophysiology, and this emphasises that the presence of HRV may be indicated by the presence of URT symptoms. Furthermore it has been reported that patients who report both cold symptoms and a sore throat have higher HRV loads than those who reported only one symptom, and that HRV loads remained elevated for longer in patients with both symptoms compared to those reporting just one symptom. These findings highlight the importance of treating HRV infection specifically, now that there is an effective method of early identification. This further emphasises the need for rapid development of antiviral treatment against HRV infection in these patients.

CHAPTER 6. HRV infection and secondary bacterial infection during COPD exacerbations

6.1 Introduction

This study aimed to explore changes in the prevalence and load of HRV and typical airway bacteria during naturally occurring COPD exacerbations and at various time-points during the recovery period.

There have been numerous studies investigating the role of bacteria or viruses in COPD but little work has explored the subject of bacterial and viral co-infection in COPD. This area is becoming of greater interest, however it is still unclear whether an initial bacterial infection leads to the development of a secondary viral infection or whether it is an initial viral infection that paves the way for secondary bacterial infection. Although few studies have explored this, those that have, generally suggest the latter (Mallia et al., 2012). Furthermore it has been suggested that co-infection of the two pathogens may be coincidental, or it may be that the presence of virus or bacteria is a bystander in the exacerbation. A study by Hutchinson and colleagues reported that 36% of virus-positive COPD exacerbations developed a secondary bacterial infection over the following 7 days (Hutchinson et al., 2007). A study by Wilkinson and colleagues in 2006 showed how the bacterial load of *H influenzae* at exacerbation, increased significantly in the presence of HRV compared to exacerbations without both pathogens (Wilkinson et al., 2006b). In 2006, Papi and colleagues showed that bacterial and/or viral infection was detected in 78% of exacerbations. When broken down it was found that 29.7% of exacerbations were

found to be positive for bacteria only, 23.4% had virus only and 25% had co-infection of both bacteria and virus (Papi et al., 2006). These studies focused on exacerbation onset, however to date, no study has explored this area throughout the whole time-course of an exacerbation and recovery period, in natural exacerbations.

In 2012, Mallia and colleagues performed an experimental a study in which moderate COPD patients and healthy controls were experimentally infected with a low dose of HRV and sampled for sputum at various time-points post-inoculation (Mallia et al., 2012). The results showed that HRV load in sputum, measured by qPCR, peaked at Days 5-9 post-inoculation but then decreased until reaching zero at Day 42. Airway bacterial load, measured by culture however, started to increase from Day 5, peaking at Day 15 before decreasing until Day 42 (Mallia et al., 2012). These results from this experimental model, suggest that airway bacterial infection is precipitated by viral infection. This study was performed in moderate COPD patients with a low dose of virus and is a valid model of human COPD exacerbations allowing measurements to be taken in a controlled manner. However, it is essential to further this experimental work and explore the role of viral and bacterial co-infection during naturally occurring exacerbation time-courses.

For the first time, this study aimed to explore changes in the prevalence and load of HRV and typical airway bacteria, during naturally occurring COPD exacerbation and recovery. Furthermore, this study aimed to determine whether an initial HRV infection at COPD

exacerbation onset leads to the development of a secondary bacterial infection during the recovery period of natural COPD exacerbations. This study was performed by splitting the collected data into two groups for analysis; firstly, exacerbations positive for typical airway bacteria at exacerbations presentation, and secondly, exacerbation negative for typical airway bacteria, at exacerbation presentation.

6.2 Characteristics of patients used in analysis of exacerbations positive for bacteria

In exacerbations positive for typical airway bacteria at exacerbation presentation (ExP), a total of 263 samples were obtained; 66 taken at ExP, 42 at Day 3, 58 at Day 7, 55 at Day 14 and 42 at Day 35 from 44 patients were analysed between April 2010 and June 2013. 94% of these exacerbations were treated with antibiotic therapy and 88% with oral steroids at exacerbation presentation. The baseline clinical characteristics for these patients are shown in Table 6.1.

FEV ₁ , litres	1.11 (\pm 0.5)
FVC, litres	2.31 (\pm 0.8)
FEV ₁ /FVC, %	46.2 (\pm 14.9)
Predicted FEV ₁ , %	44.9 (\pm 20.3)
Current Smoker, %	33
Age, years	71 (\pm 7.5)
Male gender, %	64

Table 6.1: Baseline clinical characteristics of 44 patients in the London COPD Cohort, who participated in a study of HRV and typical airway bacterial presence during naturally occurring COPD exacerbations and recovery in exacerbations positive for bacteria at presentation. Data are presented as mean (\pm SD) or percentage (%).

6.3 Results - exacerbations positive for bacteria at presentation

6.3.1 HRV and bacterial prevalence in exacerbations positive for bacteria at presentation

HRV prevalence fell from 50.0% at ExP to 22.5% at Day 3, 21.8% at Day 7, 14% at Day 14 and 5.3% at Day 35. Significant falls in HRV prevalence from ExP were seen at all subsequent time-points (all $p \leq 0.004$). Typical airway bacterial prevalence decreased from 100% at ExP to 61% at Day 3, 51.8% at Day 7, 62.7% at Day 14 and 48.8% at Day 35 as shown in Table 6.3. Significant decreases in the bacterial prevalence from ExP were seen at all following time-points (all $p < 0.001$) but no other significant differences were seen between any other time-points (Figure 6.1).

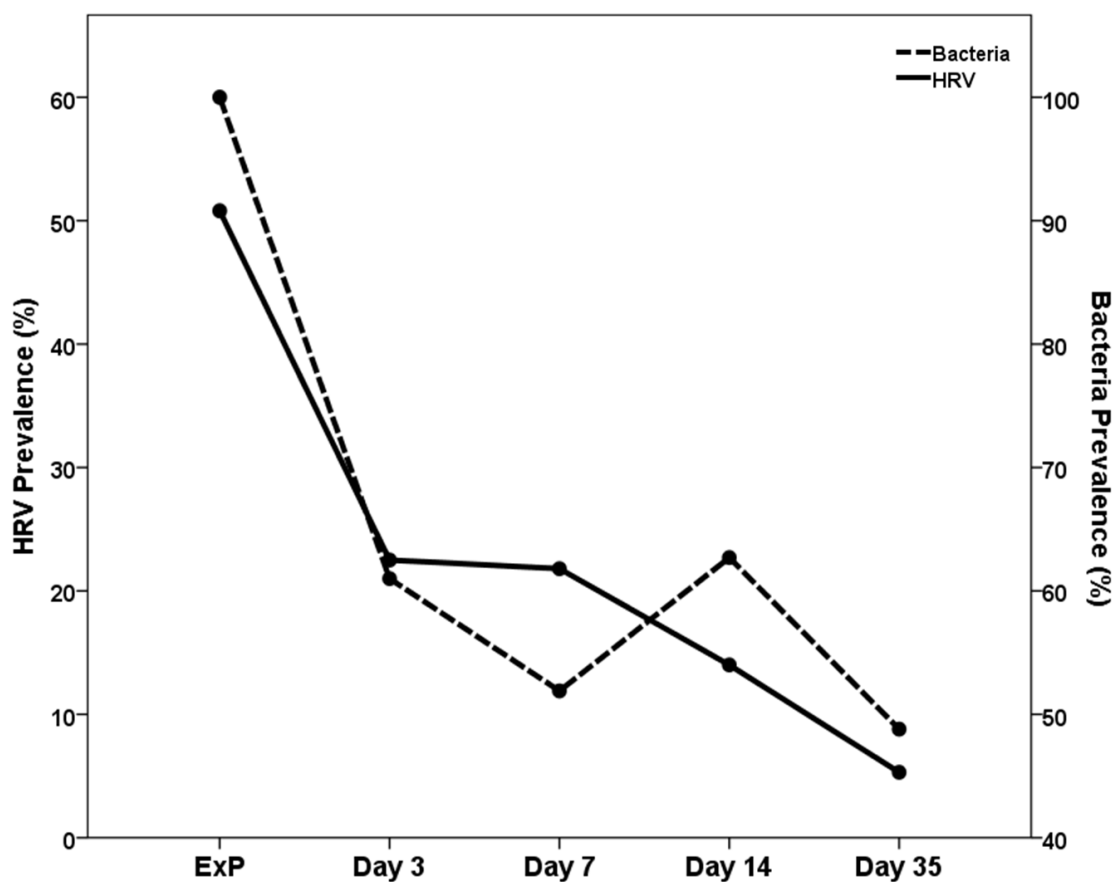


Figure 6.1: Changes in the prevalence of HRV and total typical airway bacteria over the time-course of naturally occurring exacerbations in exacerbations positive for bacteria and HRV at presentation (ExP).

Total bacterial prevalence was broken down to explore the prevalence of individual bacterial species, *H influenzae*, *M catarrhalis* and *S pneumoniae*, over the time-course of COPD exacerbation and during recovery. The prevalence of *S pneumoniae* decreased significantly from 33.3% at ExP to 12.2% at Day 3 ($p=0.014$), 16.1% at Day 7 ($p=0.029$), 15.7% at Day 14 ($p=0.030$) and 16.4% at Day 35 ($p=0.032$). There was a significant

decrease in the prevalence of *M catarrhalis* from 18.2% at ExP to 0% by Day 3 ($p=0.004$). The prevalence remained at 0% until Day 14 when the prevalence increased to 3.9% however this increase in *M cararrhalis* from Day 7 to Day 14 did not reach statistical significance ($p=0.135$). The prevalence of *H influenzae* decreased significantly from 81.8% at ExP to 51.2% at Day 3, 41.1% at Day 7, 52.9% at Day 14 and 41.5% at Day 35 (all $p<0.001$) as shown in Table 6.3. *H influenzae* prevalence was found to be significantly higher than both *M catarrhalis* and *S pneumoniae* at all time-points (all $p\leq 0.015$) (Figure 6.2).

	HRV (%)	All bacteria (%)	HI (%)	MC (%)	SP (%)
ExP	50.0	100.0	81.8	18.2	33.3
D3	22.5	61.0	51.2	0.0	12.2
D7	21.8	51.8	41.1	0.0	16.1
D14	14.0	62.7	52.9	3.9	15.7
D35	5.3	48.8	41.5	0.0	16.4

Table 6.2: Changes in the prevalence of HRV, all typical airway bacteria, and individual typical airway bacteria species over the time-course of naturally occurring exacerbations in exacerbations positive for bacteria at presentation (ExP). HI = *H influenzae*, MC = *M catarrhalis*, SP = *S pneumoniae*.

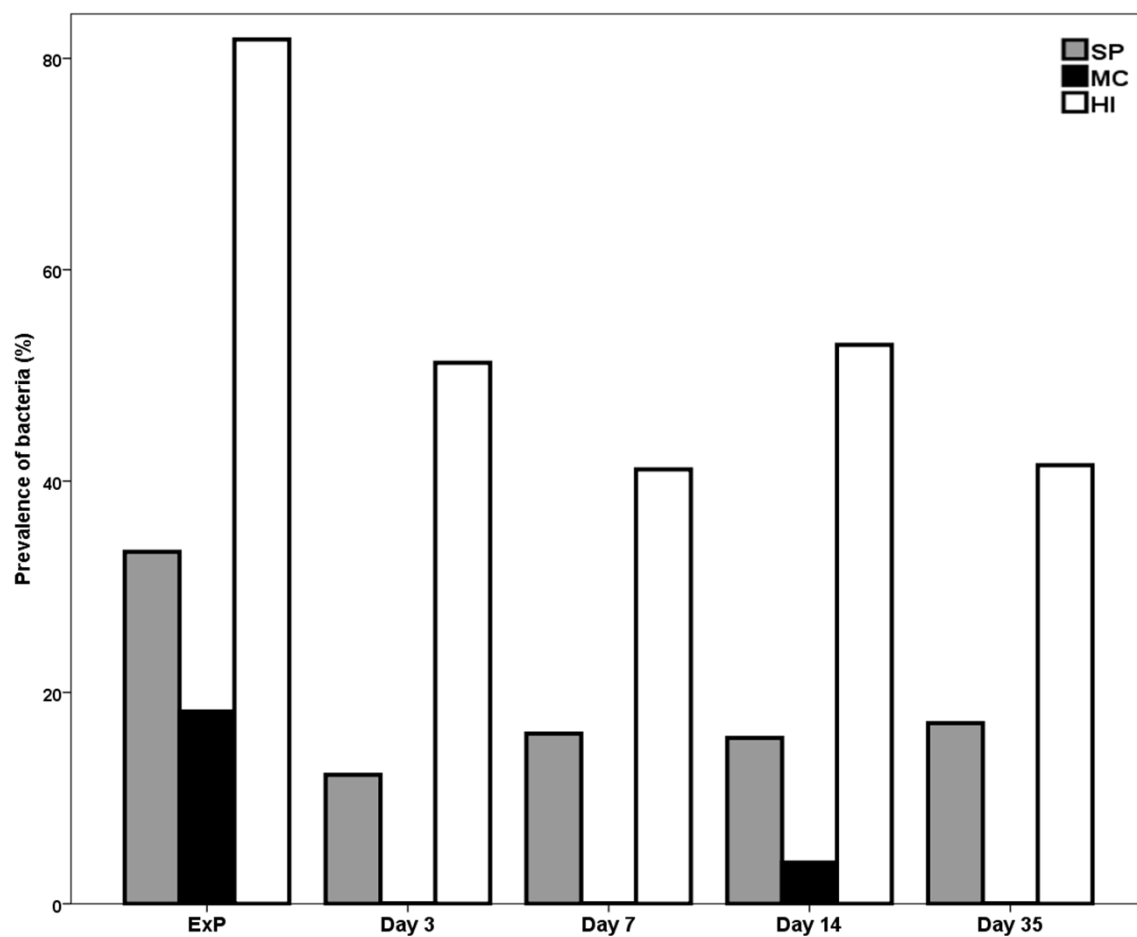


Figure 6.2: Changes in the prevalence of individual typical airway bacteria species over the time-course of naturally occurring exacerbations in exacerbations positive for bacteria at presentation (ExP). SP = *S pneumoniae* MC = *M catarrhalis* HI = *H influenzae*.

6.3.2 HRV and bacterial load in exacerbations positive for HRV and bacteria

Out of the 66 ExP samples, 33 (50%) were positive for both typical bacteria and HRV. The median (IQR) HRV load fell significantly from $10^{2.96(1.59-4.55)}$ pfu/ml at ExP to $10^{0.55(0-1.93)}$ pfu/ml at Day 3, $10^{0(0-1.42)}$ pfu/ml at Day 7 and zero by Day 14 (all $p < 0.0001$). In these 33 exacerbations, the median (IQR) bacterial load decreased significantly from $10^{7.27(6.13-8.43)}$ cfu/ml to $10^{0(0-6.43)}$ cfu/ml at Day 7 ($p < 0.001$) and then increased significantly from $10^{0(0-6.43)}$ cfu/ml at Day 7 to $10^{5.43(0-7.15)}$ cfu/ml at Day 14 ($p = 0.049$) as HRV load reached zero (Figure 6.3).

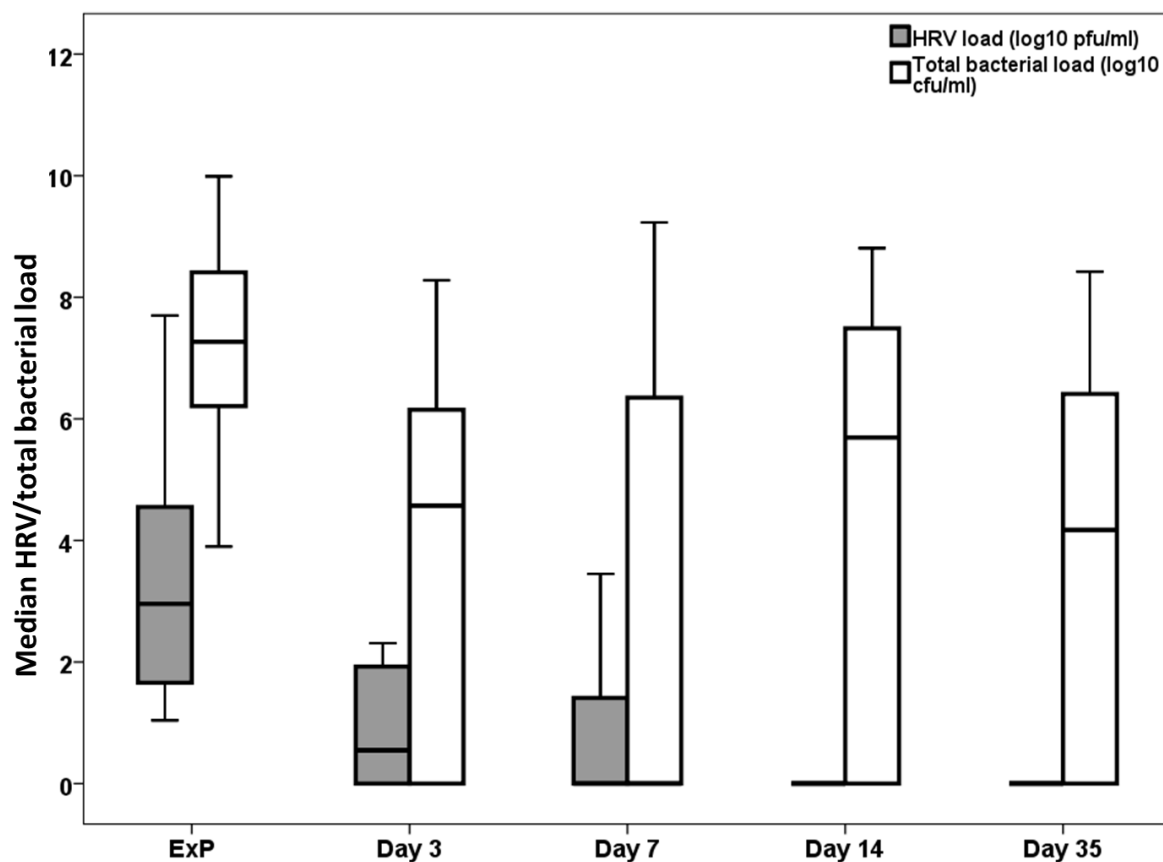


Figure 6.3: Changes in the load of HRV and typical airway bacteria over the time-course of naturally occurring exacerbations in exacerbations positive for bacteria and HRV at presentation (ExP). Data are presented as median (IQR).

Changes in HRV load and typical bacterial load were analysed using samples positive for both HRV and bacteria at all time-points in the recovery period, as well as at ExP (n=33); Day 3 (n=4), Day 7 (n=3), Day 14 (n=4) and Day 35 (n=2). Except for there being a significant decrease in the median HRV load from ExP to Day 3 (p=0.004), there were no significant differences between any of the time-points for either HRV or bacteria; this is presumably as numbers in this samples set were small.

Total bacterial load over the time-course of COPD exacerbation and recovery was subdivided to explore the changes in load of the individual bacterial species over the time period. *M catarrhalis* was not detected at any time point. For bacterial species *S pneumoniae* and *H influenzae* there was a significant decrease in the median load between ExP and Day 3 (p=0.043 and p=0.028 respectively) and between ExP and Day 7 (p=0.008 and p<0.0001, respectively). The median load of *H influenzae* increased significantly from $10^{0(0-5.86)}$ cfu/ml at Day 7 to $10^{4.37(0-6.62)}$ cfu/ml at Day 14 (p=0.049) but no difference was seen for *S pneumoniae* between these two time-points (Figure 6.5). The median load of *H influenzae* was significantly higher than that of *S pneumoniae* at every time point measured (all p<0.005) (Figure 6.4).

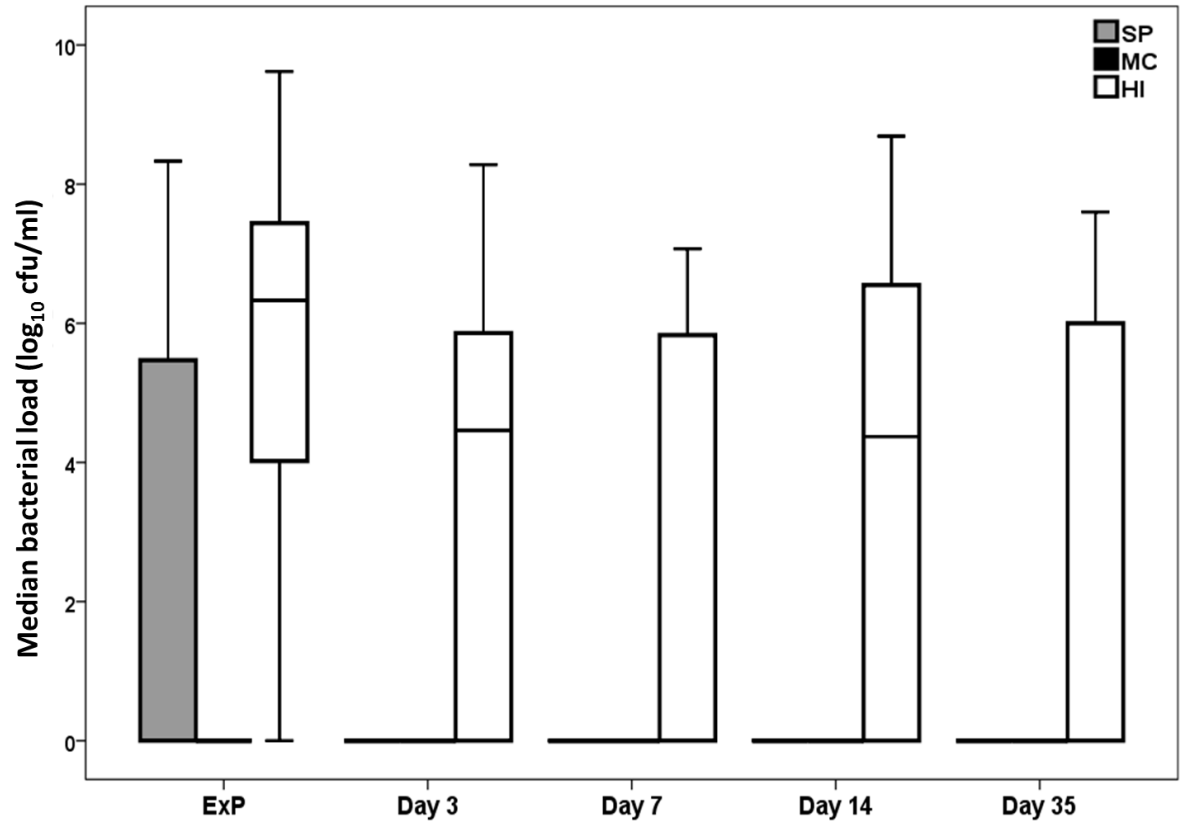


Figure 6.4: Changes in the load of the three typical airway bacteria *S pneumoniae*, *M catarrhalis* and *H influenzae* over the time-course of naturally occurring exacerbations in exacerbations positive for bacteria and HRV at presentation (Exp). Data are presented as median (IQR). SP = *S pneumoniae* MC = *M catarrhalis* HI = *H influenzae*.

As *H influenzae* was shown to be the most prevalent bacterial species at all time points, and to have the highest bacterial load at all time points, the changes in *H influenzae* and HRV over the time-course of an exacerbation in samples positive for HRV and positive for *H influenzae* at ExP were investigated. The median load of HRV fell significantly from $10^{2.96(1.59-4.55)}$ pfu/ml at ExP to $10^{0.55(0-1.93)}$ pfu/ml at Day 3 ($p<0.001$) reaching zero by Day 14. The median (IQR) *H influenzae* load decreased significantly from $10^{6.33(3.96-7.63)}$ cfu/ml at ExP to $10^{4.46(0-6.15)}$ cfu/ml by Day 3 ($p=0.028$) and $10^{0(0-5.86)}$ cfu/ml by Day 7 ($p<0.001$). The median (IQR) load increased from $10^{0(0-5.86)}$ cfu/ml at Day 7 ($p=0.048$) to $10^{4.37(0-6.62)}$ cfu/ml by Day 14 ($p=0.050$) (Figure 6.5).

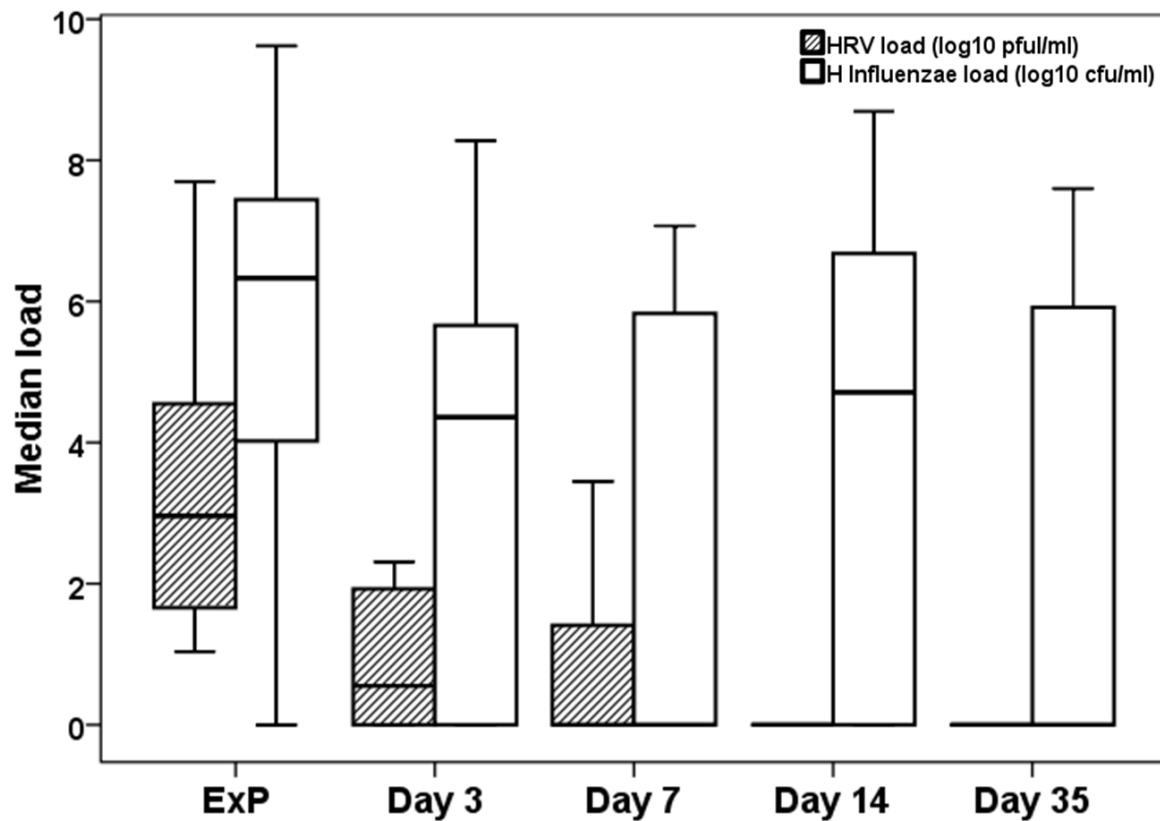


Figure 6.5: Changes in the load of HRV and *H influenzae* over the time-course of naturally occurring exacerbations in exacerbations positive for HRV and *H influenzae* at presentation (ExP). Data are presented as median (IQR).

6.4 Characteristics of patients used in analysis of exacerbations negative for bacteria at presentation

In order to verify that the significant increase in bacterial load seen at Day 14 was not due to a previous bacterial infection returning at the cessation of antibiotics, changes in bacterial prevalence and load in exacerbations negative for typical airway bacteria at exacerbation presentation were examined. A total of 220 samples were taken; 50 at exacerbation presentation, 37 at Day 3, 50 at Day 7, 47 at Day 14 and 36 at Day 35 from 41 patients were analysed between May 2010 and June 2013. All exacerbations were treated with antibiotic therapy and 72% with oral steroids at exacerbation presentation. The baseline clinical characteristics for these patients are shown in Table 6.3.

FEV ₁ , litres	1.12 (\pm 0.5)
FVC, litres	2.23 (\pm 0.9)
FEV ₁ /FVC, %	45.9 (\pm 14.7)
Predicted FEV ₁ , %	44.9 (\pm 20.3)
Current Smoker, %	33
Age, years	70.4 (\pm 8.0)
Male gender, %	61

Table 6.3: Baseline clinical characteristics of 41 patients in the London COPD Cohort, who participated in a study of HRV and typical airway bacterial presence during naturally occurring COPD exacerbation and recovery in exacerbations negative for typical bacteria at presentation. Data are presented as mean (\pm SD) or percentage (%).

6.5 Results – exacerbations negative for bacteria at presentation

6.5.1 HRV and bacterial prevalence in exacerbations negative for bacteria at exacerbation

HRV prevalence fell from 50% (25/50) at ExP to 25.7% at Day 3, 25% at Day 7, 11.6% at Day 14 and 5.9% at Day 35. Significant falls in HRV prevalence from ExP were seen at all subsequent time-points (all $p < 0.001$). Typical airway bacterial prevalence increased from 0% at ExP to 17.1% at Day 3, 17.4% at Day 7, 46.7% at Day 14 and 45.5% at Day 35 as shown in Table 6.5. Significant increases in typical airway bacteria prevalence from ExP were seen at all following time-points (all $p \leq 0.002$). There was a significant increase in bacterial prevalence from Day 7 to Day 14 ($p = 0.006$) but no significant differences were seen between any other time-points (Figure 6.6).

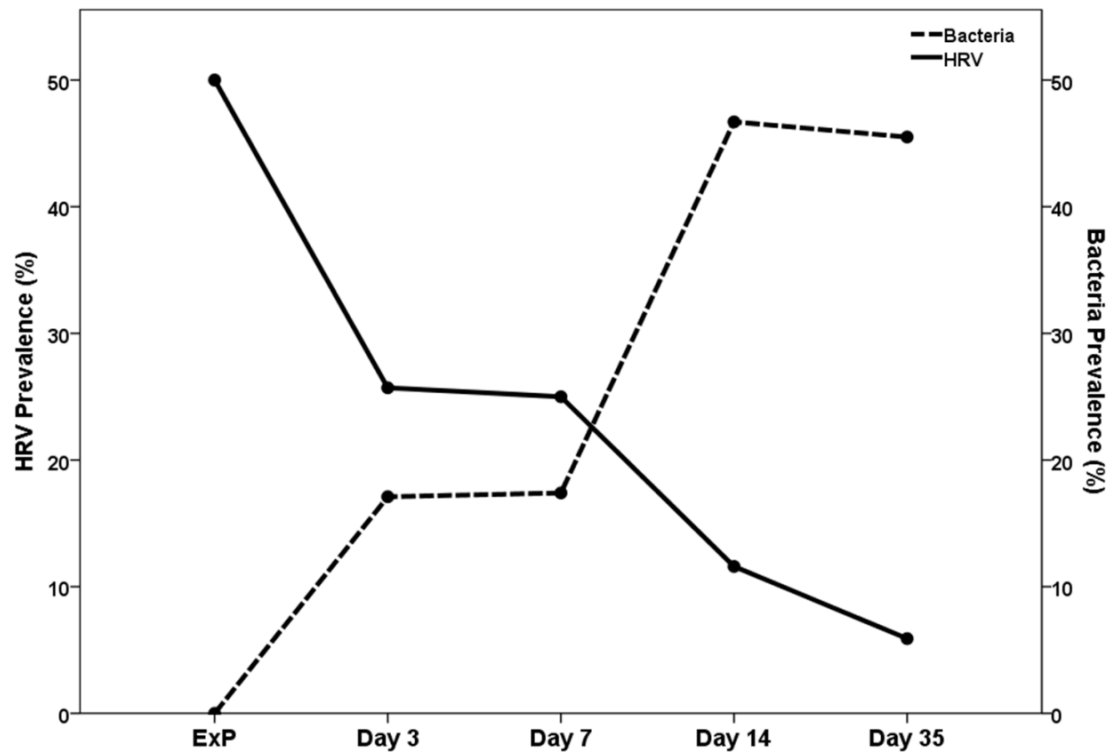


Figure 6.6: Changes in prevalence of HRV and total typical airway bacteria over the time-course of naturally occurring exacerbations in exacerbations negative for typical bacteria but positive for HRV at presentation (ExP).

Total bacterial prevalence was subdivided to explore the prevalence of individual bacterial species, *H influenzae*, *M catarrhalis* and *S pneumoniae*, over the time-course of COPD exacerbation and recovery. The prevalence of *S pneumoniae* increased significantly from 0% at ExP to 14.3% at Day 3 ($p=0.006$), 13.3% at Day 14 ($p=0.008$) and to 15.2% at Day 35 ($p=0.005$). There was a significant increase in the prevalence of *M catarrhalis* from 0% at ExP to 12.1% at Day 35 ($p=0.012$) but not at any other time point. The prevalence of *H influenzae* increased significantly from 0% at ExP to 13% at Day 7, 35.6% at Day 14 and 27.3% at Day 35 (all $p<0.024$) as shown in Table 6.4. There was a significant increase in *H influenzae* prevalence from Day 7 to Day 14 ($p=0.012$) but not for either of the other two bacteria. The prevalence of *H influenzae* was found to be significantly higher than both *M catarrhalis* ($p=0.001$) and *S pneumoniae* ($p=0.014$) at Day 14 (Figure 6.7).

	HRV (%)	All bacteria (%)	HI (%)	MC (%)	SP (%)
ExP	50.0	0.0	0.0	0.0	0.0
D3	20.7	17.1	5.7	2.9	14.3
D7	25.0	17.4	13.0	2.2	4.3
D14	11.6	46.7	35.6	6.7	13.3
D35	5.9	45.5	27.3	12.1	15.2

Table 6.4: Changes in the prevalence of HRV, all typical airway bacteria, and individual typical airway bacteria species over the time-course of naturally occurring exacerbations in exacerbations negative for bacteria at presentation (ExP). SP = *S pneumoniae* MC = *M catarrhalis* HI = *H influenzae*.

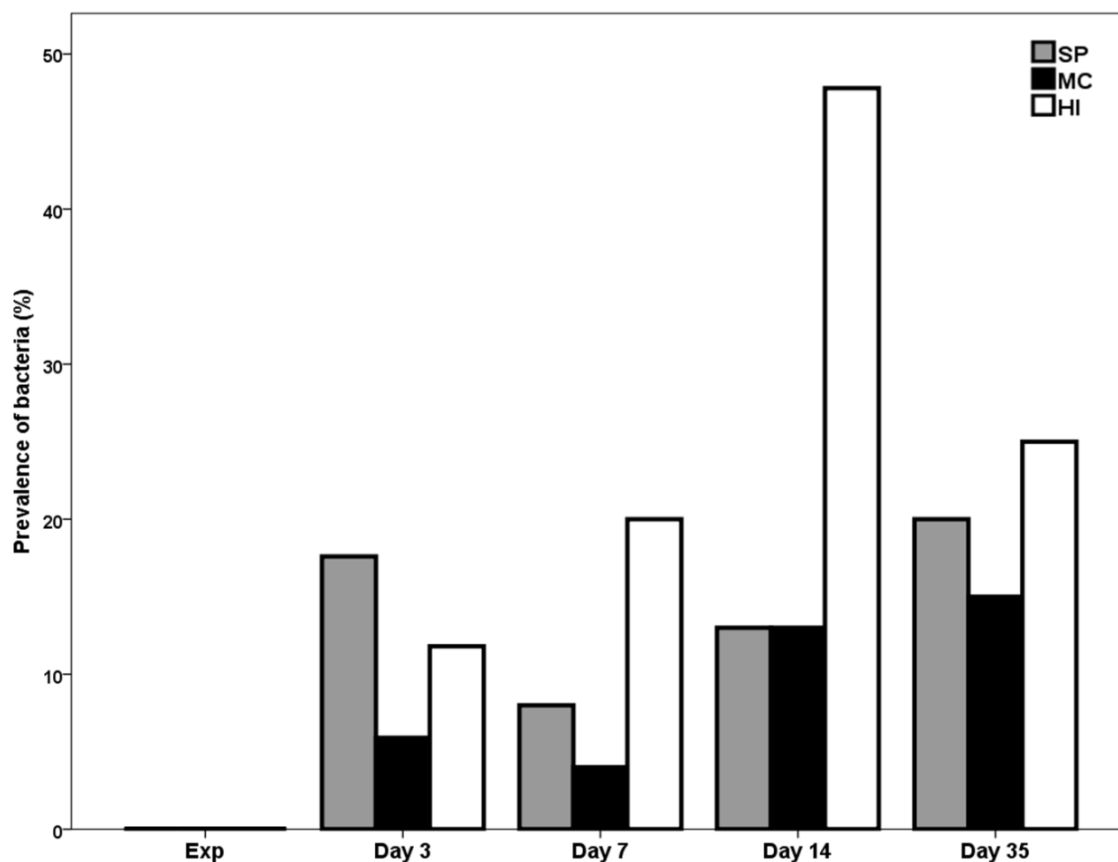


Figure 6.7: Changes in prevalence of individual typical airway bacteria species over the time-course of naturally occurring exacerbations in exacerbations negative for bacteria at presentation (Exp). SP = *S pneumoniae*, MC = *M catarrhalis*, HI = *H influenzae*.

6.5.2 HRV and bacterial load in exacerbations negative for bacteria but positive for HRV at presentation

Out of the 50 ExP samples, 25 (50%) were positive for HRV but negative for typical bacteria. The median (IQR) HRV load fell from $10^{3.17(1.65-4.92)}$ pfu/ml at ExP to zero by Day 14 ($p=0.001$). In these 25 exacerbations, the median (IQR) bacterial load increased significantly from zero at ExP to $10^{5.79(0-7.12)}$ cfu/ml by Day 14 ($p<0.001$) with 52% (13/25) of bacteria-negative samples becoming positive by Day 14 (Figure 6.8). There was a significant increase in the median total bacterial load from Day 7 ($10^{0(0-5.00)}$ pfu/ml) to Day 14 ($10^{5.79(0-7.12)}$ pfu/ml) ($p=0.038$). In absolute numbers, there was a decrease in bacterial load from Day 14 to Day 35 ($10^{0(0-6.62)}$ pfu/ml) but this fall however was not significant ($p=0.492$).

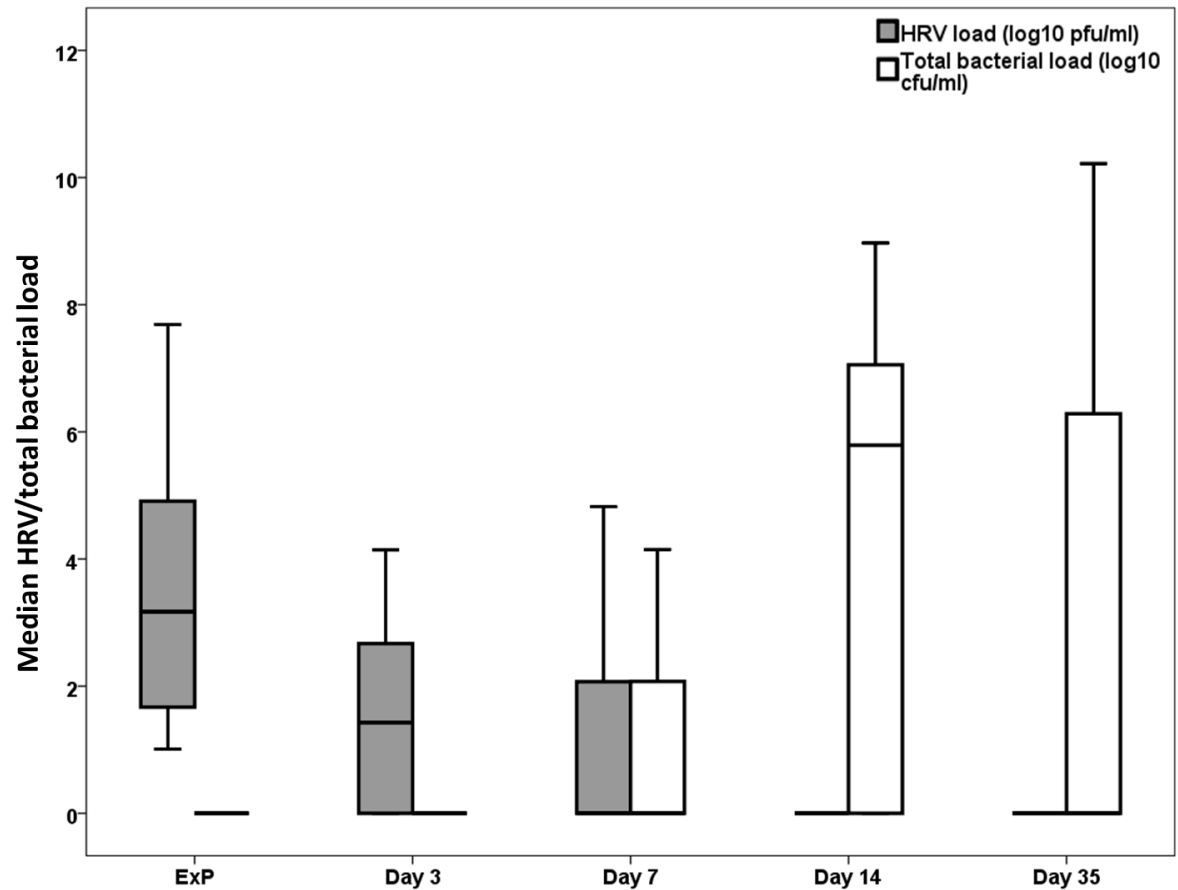


Figure 6.8: Changes in load of HRV and typical airway bacteria over the time-course of naturally occurring exacerbations in exacerbations negative for bacteria but positive for HRV at presentation (ExP). Data are presented as median (IQR).

Total bacterial load over the time-course of COPD exacerbation and during recovery was subdivided to explore the changes in the load of individual bacterial species over the time-course. For all bacterial species, *S pneumoniae*, *M catarrhalis* and *H influenzae* there were significant increases in load between ExP and Day 35 ($p=0.021$, $p=0.048$, $p=0.009$ respectively) but not between any other time points. The median (IQR) *H influenzae* load increased significantly from $10^{0(0-0)}$ cfu/ml at ExP to $10^{0(0-6.69)}$ cfu/ml at Day 14 ($p<0.001$) and from $10^{0(0-0)}$ cfu/ml at Day 7 to $10^{0(0-6.69)}$ cfu/ml at Day 14 ($p<0.037$). In absolute numbers, the median *H influenzae* load decreased from $10^{0(0-6.69)}$ cfu/ml at Day 14 to $10^{0(0-3.85)}$ cfu/ml at Day 35 however this was not significant (Figure 6.9).

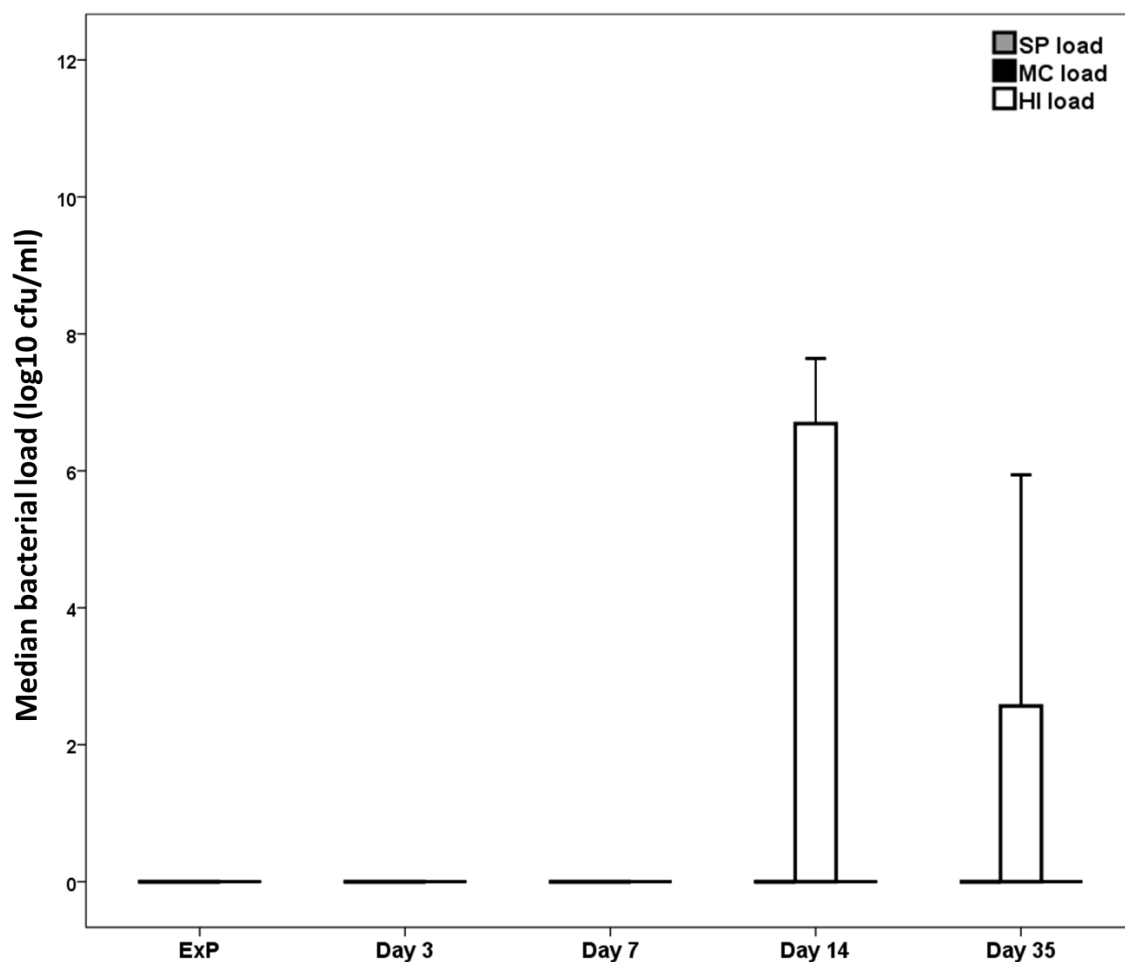


Figure 6.9: Changes in load of the three typical airway bacteria *S pneumoniae*, *M catarrhalis* and *H influenzae* over the time-course of naturally occurring exacerbations in exacerbations negative for bacteria but positive for HRV at presentation (ExP). Data are presented as median (IQR). SP = *S pneumoniae* MC = *M catarrhalis* HI = *H influenzae*.

As *H influenzae* has been shown to be the most prevalent bacterial species at Day 7 and Day 14, and to have the highest bacterial load at those time points, changes in *H influenzae* and HRV load over the time-course of an exacerbation in samples positive for HRV at ExP but negative for *H influenzae* were investigated. The median load of HRV fell significantly from $10^{3.37(1.66-4.92)}$ pfu/ml at ExP to $10^{1.24(0-2.67)}$ pfu/ml at Day 3 ($p=0.001$) reaching zero by Day 14. The median *H influenzae* load increased significantly from zero at ExP to $10^{0(0-6.69)}$ cfu/ml by Day 14 ($p<0.001$) and then decreased significantly to $10^{0(0-3.85)}$ cfu/ml at Day 35 (Figure 6.10).

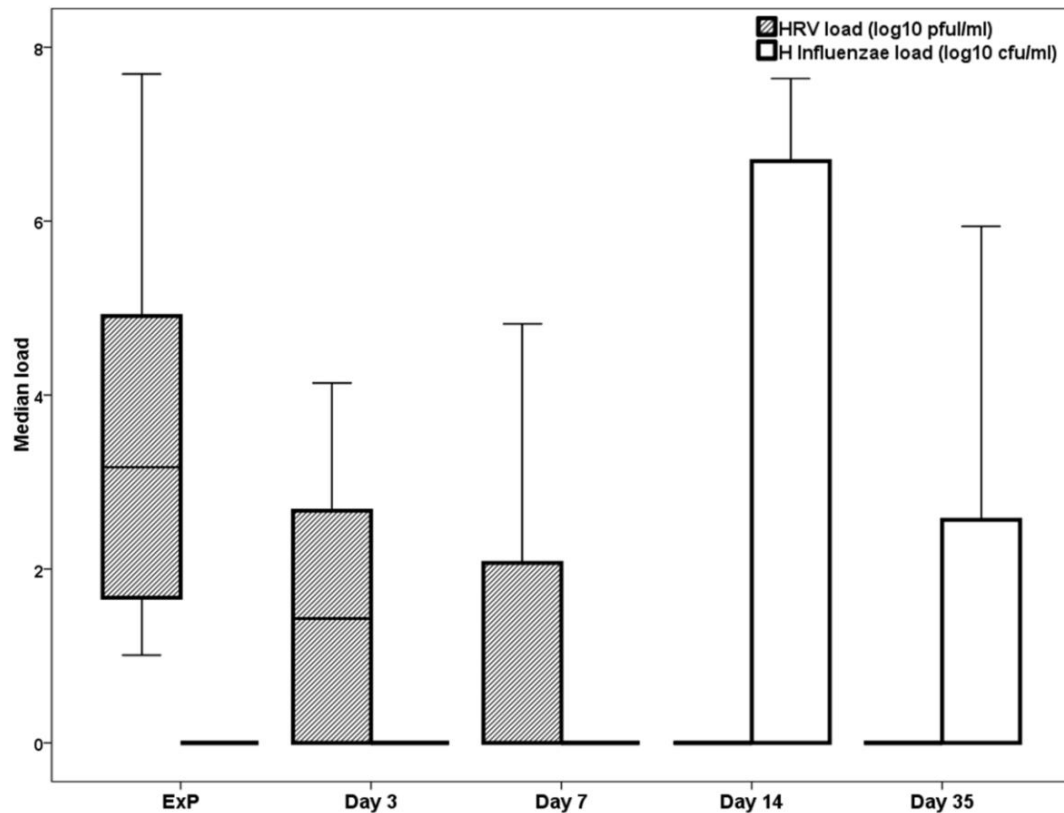


Figure 6.10: Changes in load of HRV and *H influenzae* over the time-course of naturally occurring exacerbations in exacerbations negative for *H influenzae* but positive for HRV at presentation (ExP). Data are presented as median (IQR).

6.6 Discussion

This is the first study to investigate co-infection of HRV and typical airway bacteria in COPD patients during the time-course of naturally occurring exacerbations and recovery, using qPCR techniques.

To date, only a small number of studies have explored the subject of co-infection. In 2006, Wilkinson and colleagues found an interaction between *H influenzae* and HRV infection at COPD exacerbation showing that airway bacterial load, measured using culture techniques, was markedly increased in the presence of HRV and *H influenzae*, compared to cases where HRV were not present which suggests there is an interactive effect of HRV infection with *H influenzae*, allowing greater proliferation of airway bacteria within the airways (Wilkinson et al., 2006). Papi and colleagues reported co-infection of viruses and bacteria in 25% of exacerbations (29.7% bacteria only and 23.4% virus only). They showed that exacerbations associated with infection are more severe in terms of length of hospitalisation and lung function but that exacerbations with co-infection were associated with even greater impairment in terms of lung function and longer length of hospitalisation. This suggests that co-infection is an even bigger driver of exacerbation severity, greater than that of bacteria or virus alone and expresses the importance of therapeutic strategies and the need for them to be focused on treating infection (Papi et al., 2006). In 2013, Perotin and colleagues showed that 44% of exacerbations were

positive for a virus, 42% were positive for bacteria and 27% had co-infection of viruses and bacteria. They reported that patients with co-infection did not present greater clinical severity scores at exacerbation compared to those with single infections (Perotin et al., 2013). Wark and colleagues showed virus positivity in 40% of exacerbations, the presence of bacteria in 21% and the presence of both in 9%. Conversely to the work by Perotin, it was reported that viral-associated exacerbations, in particular those co-infected with bacteria had more severe exacerbations and were more likely to be readmitted to hospital (Wark et al., 2013). A study exploring the prevalence of bacterial pathogens associated with viral infections of the respiratory tract found that in nasopharyngeal samples collected from symptomatic children, high levels of bacteria were found more commonly in the presence of RSV infections and that during an RSV or HRV infection, a single bacterial species can constitute between 80-95% of the bacterial community present (Chappell et al., 2013). In 2012 Mallia and colleagues experimentally infected moderate COPD patients with HRV and found that secondary bacterial infection developed in 60% of COPD subjects, with bacterial load peaking on Day 15 (Mallia et al., 2012). The results from the current study support these findings by Mallia and colleagues and report 52% of patients negative for bacteria at exacerbation presentation were positive by Day 14 post-exacerbation with bacterial load increasing significantly by this time-point. The results suggest that previous studies of COPD exacerbations have underestimated the rates of dual infection caused by viral and bacterial infections at different times. In order for these findings to be used in clinical practice, it is important to determine the effect of co-

infection on clinical outcomes, with the aim of treating exacerbations to account for potential secondary bacterial infection. It was shown in experimental infection that the development of a secondary bacterial infection is likely to prolong the duration of initially virus-induced COPD exacerbations (Mallia et al., 2012) however this area has not yet been explored in natural exacerbations and these results should encourage such study. Treating respiratory viral infection, in particular HRV, is crucial to improve COPD exacerbation treatment strategies. The findings from this study should encourage the development of novel therapeutic targets for HRV.

In exacerbations positive for typical airway bacteria at presentation, the bacterial load decreased significantly from presentation to Day 7 but then significantly increased again by Day 14. It could be argued that this finding is due to the cessation of antibiotics as 94% of these exacerbations were treated with 7 days of antibiotics at exacerbation presentation (one was treated for 10 days). Antibiotic therapy reduces total bacterial load; the exacerbations in the current study were almost exclusively treated with a 7-day course and the results show a significant decrease in total bacterial load by Day 7 compared to exacerbation presentation. The significant increase in bacterial load from Day 7 to Day 14 may be explained by antibiotic cessation at Day 7, if bacterial growth increased after antibiotic therapy was discontinued. However, in exacerbations negative for bacteria at presentation, the total bacterial load was significantly increased at Day 14 compared to presentation even though all of these exacerbations were also treated with 7 days of

antibiotic therapy at exacerbation presentation. This finding suggests a genuine link between HRV infection and the development of a secondary bacterial infection, rather than bacterial load increasing at Day 14 solely because of antibiotic cessation.

The fact that all of the exacerbations that were negative for bacteria at presentation and became positive by Day 14 were treated with antibiotics, suggests that in exacerbations with an initial viral infection followed by a secondary bacterial infection, antiviral therapy at exacerbation may be more effective than antibiotics at reducing or eradicating viral infection and may prevent the development of a secondary bacterial infection. It is more likely, however, that antibiotics may be beneficial in the latter part of the exacerbation when a secondary bacterial infection may develop, so in these cases, both therapies may be required. Additionally, lengthening the duration of antibiotic treatment may prevent secondary infection. Patients in this study were given antibiotics for 7 days. Increasing this to 14 days may be a way of suppressing bacterial growth after or during HRV infection however to date there is no evidence that this would be the case. Furthermore, the potential benefits of extending a course of antibiotics or giving multiple antibiotic courses, must be balanced against the risk of side effects associated with this.

When exploring the changes in prevalence and load of individual bacterial species over the time-course of exacerbation onset and recovery, *H influenzae* was found to be the most prevalent species and was found at higher loads than both *S pneumoniae* and *M*

catarrhalis. This finding supports previous work evaluating the prevalence and load of *S pneumoniae*, *M catarrhalis* and *H influenzae* in the stable state and at COPD exacerbation which showed *H influenzae* to be the most prevalent bacterial species at both disease states (Garcha et al., 2012; Hill et al., 2000; A. J. White et al., 2003) and studies showing *H influenzae* to be the most common bacterial cause of exacerbations (Murphy, 2006). In the current study, *H influenzae* load increased most significantly from Day 7 to Day 14 post-exacerbation compared to *S pneumoniae* and *M catarrhalis*, when HRV infection became undetectable. *H influenzae* was also found to have a higher prevalence and load than both *S pneumoniae* and *M catarrhalis* at Day 14 post-exacerbation suggesting *H influenzae* is the main bacterial species involved in the development of secondary bacterial infection. This finding supports the work by Wilkinson and colleagues who found that the load of typical airway bacteria increased significantly in the presence of HRV and *H influenzae* at COPD exacerbation compared to the load without HRV (Wilkinson et al., 2006).

In two major clinical trials in which the effects of ICS in COPD were evaluated (TORCH and INSPIRE trials) it was found that ICS usage was related to a higher rate of pneumonia development in COPD patients than that seen with other treatments (Calverley et al., 2011, 2007; Wedzicha et al., 2008). This association between the use of ICS therapy and the rate of pneumonia suggests that the use of steroids can play a role in the development of bacterial infection. Furthermore, it was shown in 2012 that higher bacterial load was

significantly related to higher inhaled corticosteroid dosage in stable COPD (Garcha et al., 2012) which suggests the use of ICS therapy could lead to the amplification of secondary bacterial infection. In the current study, 88% of exacerbations positive for typical airway bacteria and HRV at presentation were treated with oral steroids and 72% of exacerbations negative for typical airway bacteria and positive for HRV at presentation were treated for a median of 7 days. There was no difference in the bacterial or HRV load at any time point in the time-course between exacerbations treated with oral steroids compared to those not treated. This makes the suggestion of HRV paving the way for secondary bacterial infection, plausible, and not just due to the cessation of oral steroids after day 7. Despite this, the potential role of oral steroids in the development of secondary bacterial infection must be considered.

The data from the current study shows that secondary bacterial infection occurs after initial HRV infection however the mechanism by which this occurs is unknown. A number of hypotheses have been suggested in order to explain the mechanism. Sajjan and colleagues suggested that HRV facilitates the transmigration of bacteria across the airway epithelium allowing bacterial infection to develop, (Sajjan et al., 2008) whether a *de novo* infection or one from colonising bacteria already in the airway. Similarly, HRV infection may impair the innate immune response in alveolar macrophages and thereby provide an environment that allows the proliferation of colonising airway bacteria or the development of a new infection (Oliver et al., 2008). It has been suggested that HRV may

increase the susceptibility of bacterial adherence to airway epithelial cells; a study by Ishizuka and colleagues in 2003 showed that HRV infection stimulated *S pneumoniae* adhesion to airway epithelial cells via binding to the receptor, platelet activating factor receptor (PAF-R) (Ishizuka et al., 2003). Determining the predominant mechanism requires further study perhaps using more sophisticated techniques such as bacterial sequencing.

Exacerbation recurrence is an important concept in COPD. Hurst and colleagues showed in 2008 that exacerbations are not random events and cluster together in time such that there is a high-risk period for recurrent exacerbation in the 8-week period after the initial exacerbation (Hurst et al., 2009). The mechanisms of exacerbation recurrence are unknown, but possible suggestions such as persistence of an existing organism or the acquisition of a new pathogen have been proposed (Hurst et al., 2009). Similarly the idea of persistent inflammation in these patients has been hypothesised (Hurst et al., 2009). It was shown in 2006 that heightened systemic inflammation is associated with recurrent exacerbations within 50 days and that patients who had a recurrent exacerbations had significantly higher levels of CRP at 14 days post-initial exacerbation compared to those that did not suffer a recurrent exacerbation (Perera et al., 2007). Systemic corticosteroids given at exacerbation have been shown to be effective in accelerating the recovery of FEV₁ (Davies et al., 1999). However if they fail in suppressing inflammation, recurrent exacerbations are a potential risk (Hurst et al., 2009). Results from the current study suggest a possible mechanism of exacerbation recurrence involving the role of secondary

bacterial infection. This data shows that bacterial load peaks at Day 14 post-exacerbation as HRV infection disappears, which could trigger a second exacerbation. If this is to be the case, it is even more crucial that the initial exacerbation is treated rapidly in order to minimise the risk of a recurrent exacerbation developing.

Other studies investigating co-infection of viruses and bacteria at COPD exacerbation found a lower incidence of co-infection than the current study. This may be due to the larger number of samples collected from patients during each exacerbation in this study compared to others. A study by Papi in 2006, showed 25% co-infection at exacerbation however only one sample was taken at exacerbation (Papi et al., 2006). Similarly, Perotin showed in 2013 that 27% of exacerbation samples were positive for co-infection with only one sample per patient (Perotin et al., 2013). Hutchinson and colleagues collected two samples during exacerbation and reported that 36% of exacerbations in which a virus was detected at onset went on to develop a secondary bacterial infection over the following week. They also reported that 78% of exacerbations positive for *H influenzae* were preceded by viral symptoms (Hutchinson et al., 2007). In the current study, multiple samples were collected during exacerbations and a higher incidence of co-infection was detected than the studies described above. This suggests that repeated sampling over the duration of an exacerbation is more likely to identify the presence of co-infection.

This study was limited to HRV infection and although other viruses have been considered in other studies, HRV has been shown to be the most commonly detected at COPD exacerbation (Seemungal et al., 2001). Similarly, this study was limited to three typical airway bacteria detected at COPD exacerbation although these have been shown to be the main three detected at COPD exacerbation and it is likely that they are the main contributors in the aetiology of exacerbations.

These findings reinforce the urgent need for the rapid development of antiviral therapies as they may not only reduce the severity of an exacerbation or prevent-virus induced exacerbations, but may also have the potential to prevent secondary bacterial infections occurring.

6.7 Conclusion

Secondary bacterial infection is common after an initial HRV infection and has important implications in terms of therapy and exacerbation recurrence. Treating viral infection at exacerbation onset is crucial in terms of exacerbation length, severity and recurrence. The unmet need for rapid development of therapeutic targets for the prevention and treatment of HRV infection in COPD patients must be addressed.

CHAPTER 7. HRV infection and patient reported outcomes in patients with COPD

7.1 Introduction

This study aimed to explore associations between the EXACT questionnaire, the CAT questionnaire, and exacerbation frequency, with HRV infection, at exacerbation presentation and during the recovery period in natural exacerbations.

Patient reported outcome measures (PROs) are reports that come directly from patients giving information about how they feel or function in relation to a particular health condition and its therapy. PROs are an important element of in many research areas including COPD. In the London COPD cohort patients are followed prospectively, being reviewed every 3 months in the stable state, seen early at exacerbation onset and at 5 time-points during the recovery period. At each clinic visit, patients in the cohort undergo a series of measurements prior to treatment. These include spirometry, sputum sampling and venous blood sampling in order to measure the levels of certain blood inflammatory markers. Patients are also asked to fill in a variety of questionnaires including the “exacerbation of chronic pulmonary disease tool” (EXACT) and “COPD assessment test” (CAT). These are important patient reported outcome measures that provide information on the severity, duration and frequency of COPD exacerbations.

7.1.1 EXACT

Measuring the severity of an exacerbation is an important outcome measure in COPD. Daily diary cards used to record exacerbation symptoms are able to accurately detect the onset of exacerbations, either reported or unreported, however most daily diary cards are not sensitive enough to measure the severity of an exacerbation (Mackay et al., 2014). The EXACT is a daily symptom diary designed to capture the severity of COPD exacerbations (Leidy et al., 2011). It is comprised of 14 questions that assess attributes such as sputum, cough, chest symptoms, tiredness, anxiety and breathlessness. The majority of questions are answered by choosing one of five answers (two of the questions have 6 answer options). The answers are converted to a 0-100 scale with higher total EXACT scores indicating more symptomatic exacerbations. It has been shown that EXACT scores are sensitive to changes that occur during the recovery period after exacerbation onset (Leidy et al., 2011) however to date, no published data have examined the relationship between EXACT scores and HRV infection during COPD exacerbations and recovery. As HRV has been shown to be the most commonly detected virus at COPD exacerbation (Seemungal et al., 2001), it is important to establish whether there is an association between HRV infection and the EXACT questionnaire. In this study, patients filled in an EXACT questionnaire at each clinic visit (exacerbation and during recovery) based on the symptoms they felt and experienced that day.

7.1.2 CAT

The CAT is a short and simple questionnaire intended to measure the health status of COPD patients and the impact that COPD has on their quality of life in an objective manner (Jones et al., 2009). It is a validated 8-item questionnaire that is designed to assess and quantify the impact of COPD symptoms on patient health status (Jones et al., 2009). Like the EXACT, the CAT can be self-administered by patients and used in an ongoing manner. Patient recognition of exacerbation symptoms and prompt treatment improves exacerbation recovery and reduces further risk of hospitalisation in COPD patients (Wilkinson et al., 2004). Comparing different CAT scores for a particular patient at different time-points may provide valuable information on the impact of their disease on their quality of life, long-term. Each item in the questionnaire contains two opposing statements. Patients decide where on a scale of 0-5, between the two statements, they fit. The scores from the 8 items are summed to give a final score out of 40 to indicate disease impact without the need for complex calculation (Jones et al., 2009). A higher CAT score indicates worse health status, and exploring answers to individual questions in the questionnaire can provide insight into the influence that individual components of COPD may have on patient quality of life (Jones et al., 2009). It has previously been shown that the CAT questionnaire provides a reliable score of exacerbation severity and that CAT scores are increased at exacerbation compared to the stable state (Mackay et al., 2012). It has also been shown to reflect severity in terms of lung function and duration of

exacerbations (Mackay et al., 2012). As with the EXACT, in this study, patients filled in the CAT questionnaire at each clinic visit based on how they were feeling on that day.

7.1.3 Exacerbation frequency

There is considerable interest in the frequent exacerbator phenotype with many studies exploring exacerbation frequency among COPD patients. It has been shown that exacerbations of COPD impact on quality of life (Seemungal et al., 1998), accelerate decline in lung function (Donaldson et al., 2002), lead to an increase in mortality (Soler-Cataluña et al., 2005) and are associated with increases in airway and systemic inflammation (Hurst et al., 2006). Thus exacerbations are important events in COPD and their prevention is a key element in the management of COPD.

To date, it has been shown that exacerbations become more frequent and more severe as COPD severity increases and that the most important determinant of frequent exacerbations, is a history of exacerbations (Hurst et al., 2010). Furthermore it has been shown that exacerbations cluster together in time with a high-risk period for recurrent exacerbation in the first 8-weeks after an initial exacerbation, suggesting that exacerbations are not random events (Hurst et al., 2009).

The majority of COPD exacerbations are associated with infection (Hurst and Wedzicha, 2007) and it has been suggested that the mechanism of exacerbation recurrence is due to the persistence of an existing organism or to the acquisition of a new organism. The failure to eradicate bacteria with exacerbation therapy has been shown to be associated with incomplete recovery of inflammatory markers (A J White et al., 2003). From this, it has been suggested that exacerbation recurrence and therefore exacerbation frequency is related to persistence of inflammation (Perera et al., 2007) likely due to infection, however the association of viral infection, in particular HRV, and exacerbation frequency over the time-course of natural COPD exacerbation and recovery has not yet been explored.

The impact of HRV on patient health status is an important aspect to consider in exacerbations of COPD, and treating patients appropriately with the aim of reducing exacerbations is essential. So for the first time, this study aimed to explore the relationship of HRV with the EXACT questionnaire, with the CAT questionnaire, and with exacerbation frequency, at exacerbation presentation and during the recovery period in natural exacerbations.

7.2 Characteristics of patients used for EXACT analysis

Of the 537 time-course sputum samples, 330 had available EXACT data. The 330 samples from 57 patients were analysed between April 2010 and June 2013 as part of the sample collection performed in the London COPD cohort. These 330 samples were taken at 6 different time-points during exacerbation and recovery; 90 samples were taken at exacerbation presentation (ExP), 55 at Day 3 post-exacerbation, 70 at Day 7, 60 at Day 14, 42 at Day 35 and 13 at Day 56. The baseline clinical characteristics for these patients are shown in Table 7.1.

FEV ₁ , litres	1.28 (\pm 0.5)
FVC, litres	2.24 (\pm 0.8)
FEV ₁ /FVC,	0.49 (\pm 0.15)
Predicted FEV ₁ , %	48.9 (\pm 19.6)
Current Smoker, %	32
Age, years	72.4 (\pm 7.6)
Male gender, %	63

Table 7.1: Baseline clinical characteristics of 57 patients in the London COPD Cohort, who participated in a study of HRV infection and EXACT scores. Data are presented as mean (\pm SD) or percentage (%).

7.3 Results – EXACT

The mean(\pm SEM) EXACT scores decreased significantly from 51.1(\pm 1.11) at exacerbation presentation (ExP) to Day 3, 7, 14 and 35 during the recovery period (all $p \leq 0.010$). The mean EXACT score then increased from 40.6(\pm 1.10) at Day 35 to 46.2(\pm 2.31) at Day 56 ($p=0.025$) (Figure 7.1).

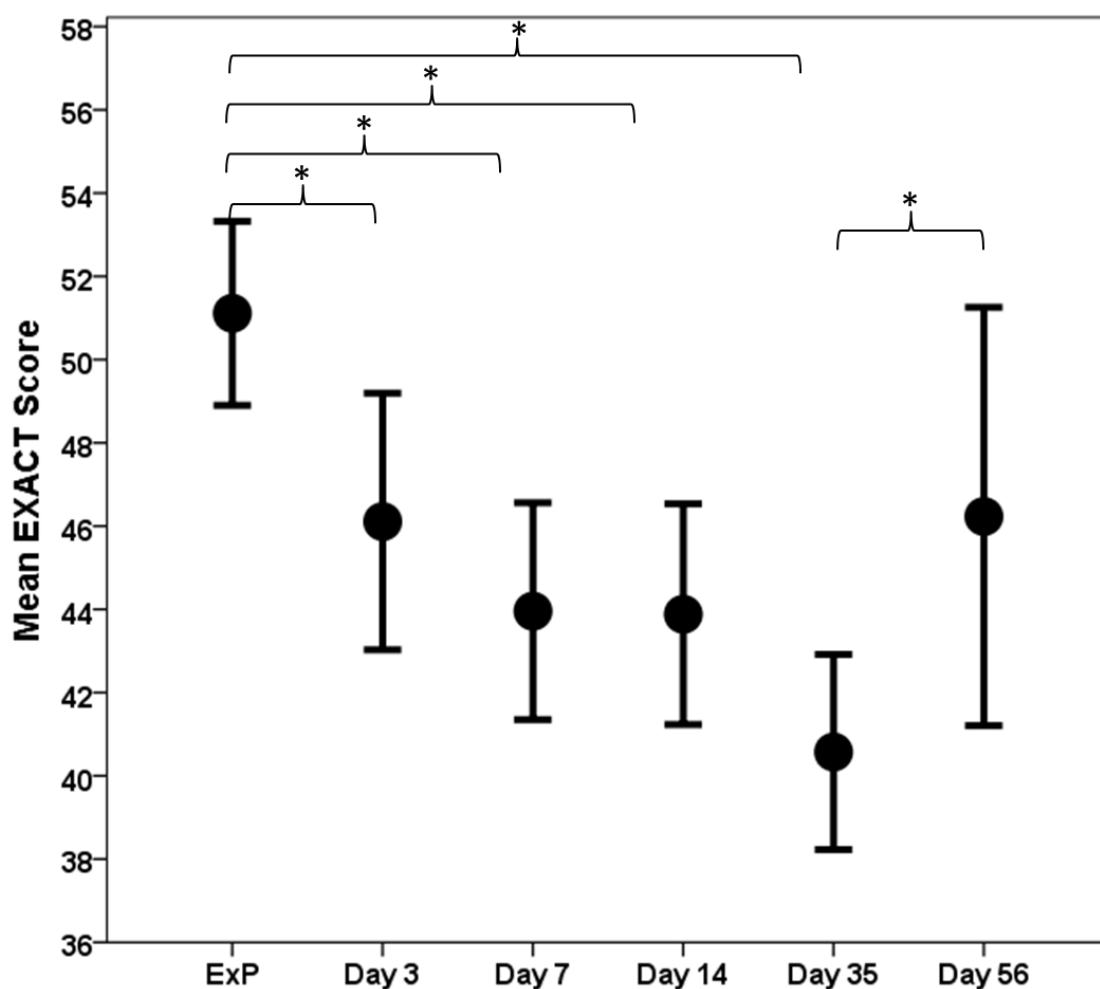


Figure 7.1: Change in EXACT scores during COPD exacerbation and recovery period. Data shown as mean (\pm 95% CI).

When exploring the changes in EXACT scores in the presence of HRV infection, it was found that samples positive for HRV (n=90) had a significantly higher mean EXACT score than samples that were not found to be positive for HRV (n=240) (Figure 7.2). In HRV-positive samples, the mean EXACT score was $48.4(\pm 1.33)$ which was significantly higher than the mean EXACT score in samples negative for HRV, $44.9(\pm 0.64)$ ($p=0.010$). Samples at all time-points were included in the analysis.

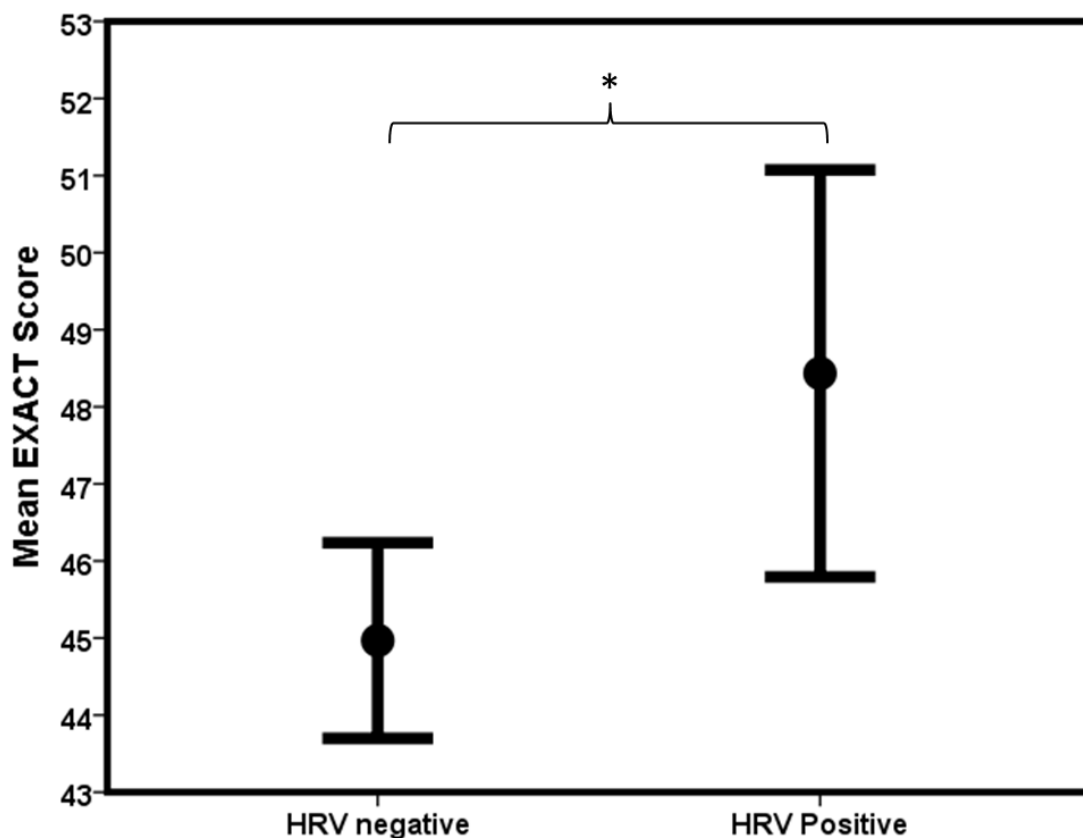


Figure 7.2: Comparison of mean EXACT scores in samples positive for HRV (n=90) and samples negative for HRV (n=240); all time-points included * $p=0.010$.

The mean EXACT scores were highest at ExP and decreased over the time-course of exacerbation recovery until Day 35, as did HRV load. There was a significant fall from ExP to Day 3 for both variables (both $p<0.010$) (Figure 7.3).

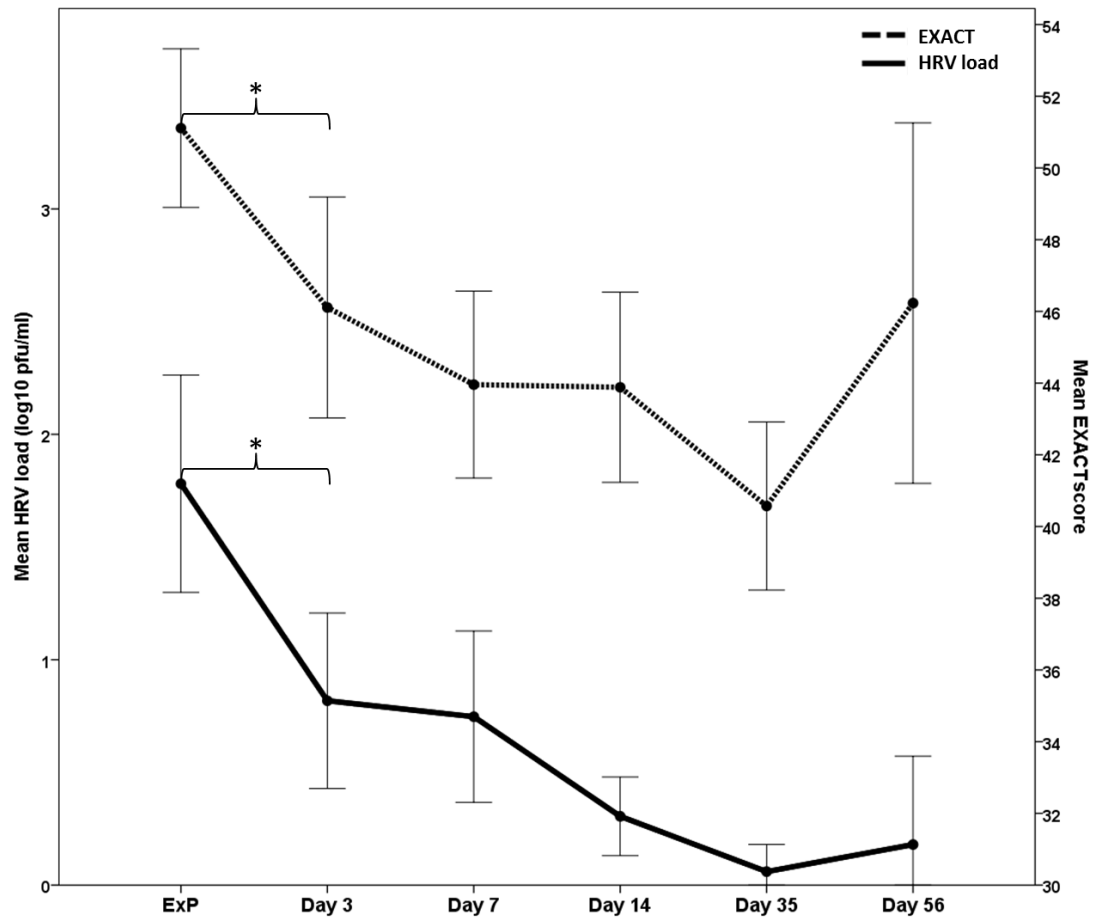


Figure 7.3: Change in EXACT scores and HRV load over the time-course of COPD exacerbation and recovery; * $p<0.010$.

There was a significant relationship between HRV load and EXACT scores with EXACT scores increasing as HRV load increased. All time-points included; $r=0.208$, $p=0.011$ (Figure 7.4).

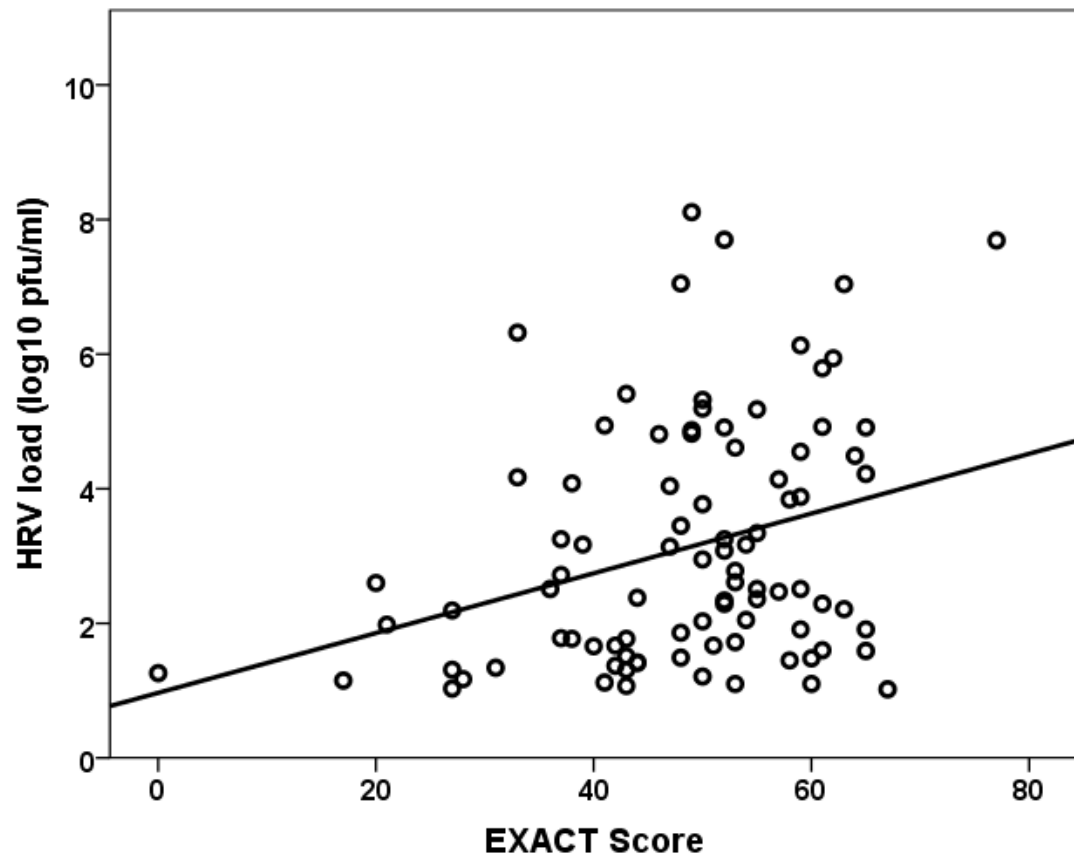


Figure 7.4: Relationship of EXACT scores and HRV load over the time-course of COPD exacerbation and recovery. All time-points included; $r=0.208$, $p=0.011$.

The EXACT questionnaire assesses lower respiratory tract symptoms and attributes such as cough, sputum production, wheeze and breathlessness as well as tiredness, sleep quality, and anxiety. As described in Chapter 5, HRV infection is associated with the presence of upper respiratory tract (URT) symptoms such as cold symptoms and sore throats. It was shown that HRV prevalence and load were higher at certain time-points during exacerbation recovery in patients who reported URT symptoms at the time of sampling, compared to those that did not.

The severity of a COPD exacerbation is one of the attributes the EXACT is designed to measure, so exploring any differences in EXACT scores between patients who reported URT symptoms and those that did not, requires investigation.

In samples associated with cold symptoms at the time of sampling (n=127), the mean EXACT score was found to be 49.8(\pm 1.02) which was significantly higher than in those that were not associated with cold symptoms at the time of sampling (n=203), 43.5 (\pm 0.68); $p < 0.001$. All time-points included (Figure 7.5).

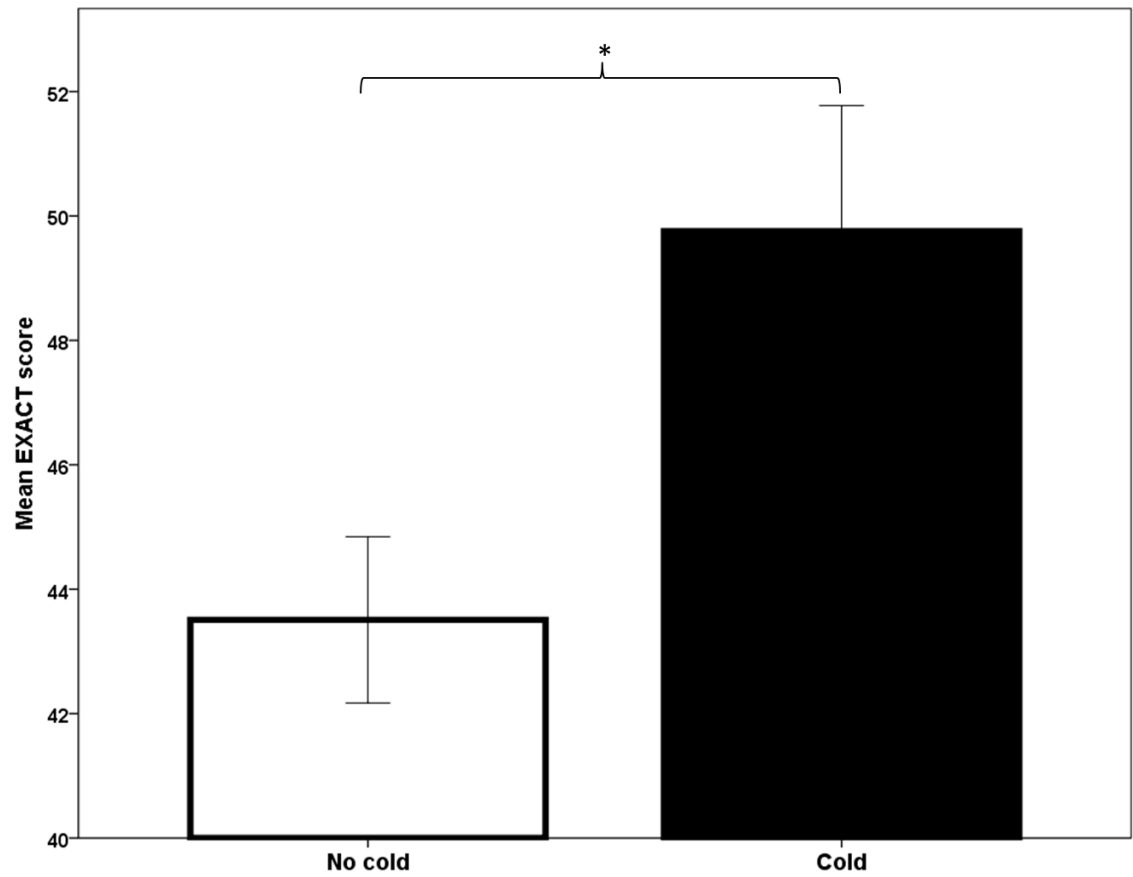


Figure 7.5: Comparison of EXACT scores in patients that reported cold symptoms at the time of sampling (n=127) and those that did not (n=203); All time-points included *p<0.001.

Similarly, in samples associated with a sore throat at the time of sampling (n=50), the mean EXACT score was found to be 50.4(\pm 1.42) which was significantly higher than in those not associated with a sore throat at the time of sampling (n=280), 45.1 (\pm 0.65); p=0.021. All time-points included (Figure 7.6).

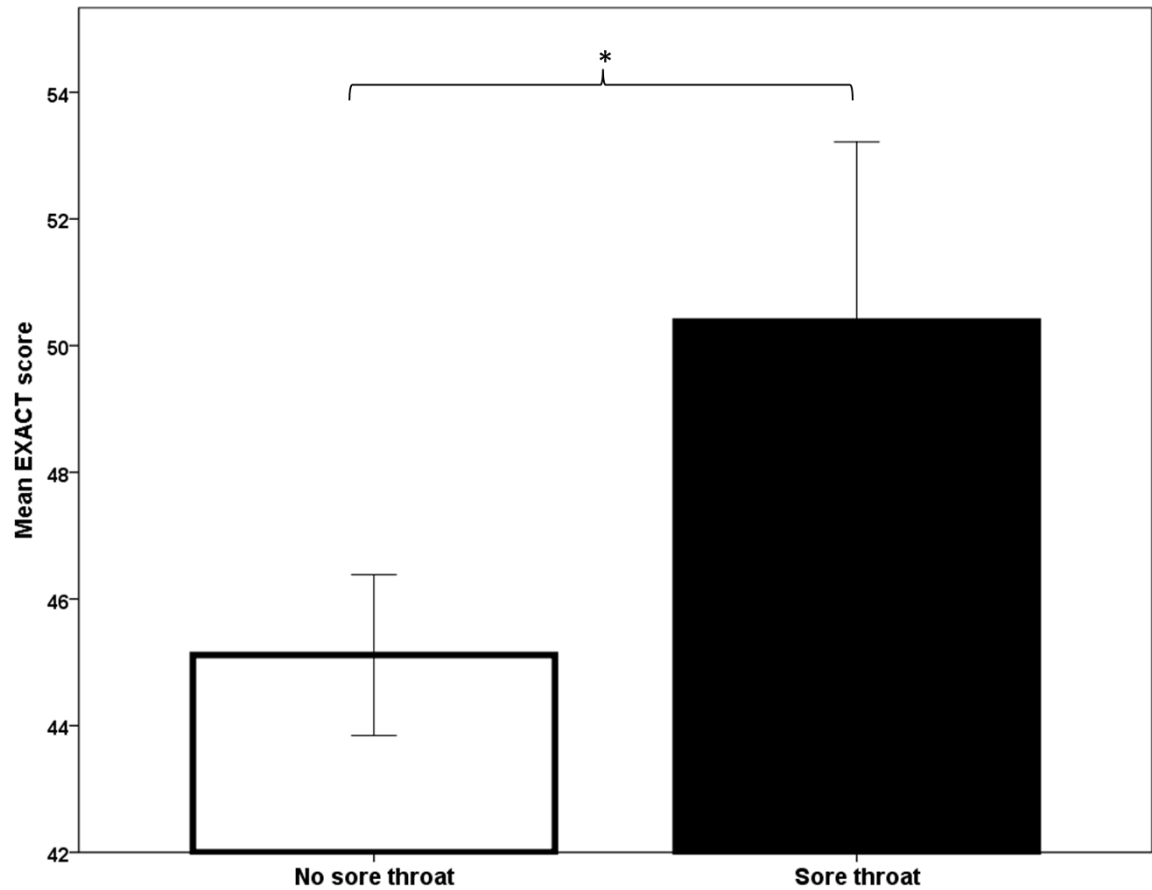


Figure 7.6: Comparison of EXACT scores at all time-points in patients that reported having a sore throat at the time of sampling (n=50) and those that did not (n=280); *p=0.021.

7.4 Characteristics of patients used for CAT analysis

Of the 537 time-course sputum samples, 388 had available CAT data. The 388 samples from 68 patients were analysed between April 2010 and June 2013 as part of the sample collection performed in the London COPD cohort. These 388 samples were taken at 6 different time-points during exacerbation and recovery; 103 samples were taken at exacerbation presentation (ExP), 69 at Day 3 post-exacerbation, 84 at Day 7, 71 at Day 14, 48 at Day 35 and 13 at Day 56. The baseline clinical characteristics for these patients are shown in Table 7.2.

FEV ₁ , litres	1.28 (\pm 0.5)
FVC, litres	2.23 (\pm 0.9)
FEV ₁ /FVC,	0.46 (\pm 0.14)
Predicted FEV ₁ , %	48.9 (\pm 19.6)
Current Smoker, %	33
Age, years	72.9 (\pm 7.6)
Male gender, %	63

Table 7.2: Baseline clinical characteristics of 68 patients in the London COPD Cohort, who participated in a study of HRV infection and CAT scores. Data are presented as mean (\pm SD) or percentage (%).

7.5 Results - CAT

The mean(\pm SEM) CAT scores decreased significantly from 22.9(\pm 0.84) at ExP to Day 7, 14 and 35 during the recovery period (all $p < 0.015$). The mean CAT score also decreased significantly from 22.1(\pm 0.88) at Day 3 to 19.9(\pm 0.87) at Day 7, 19.1(\pm 0.94) at Day 14 and 17.3(\pm 0.95) at Day 35 (all $p \leq 0.003$). There was also a significant decrease in mean CAT score from Day 7 to Day 35 ($p = 0.046$) (Figure 7.7).

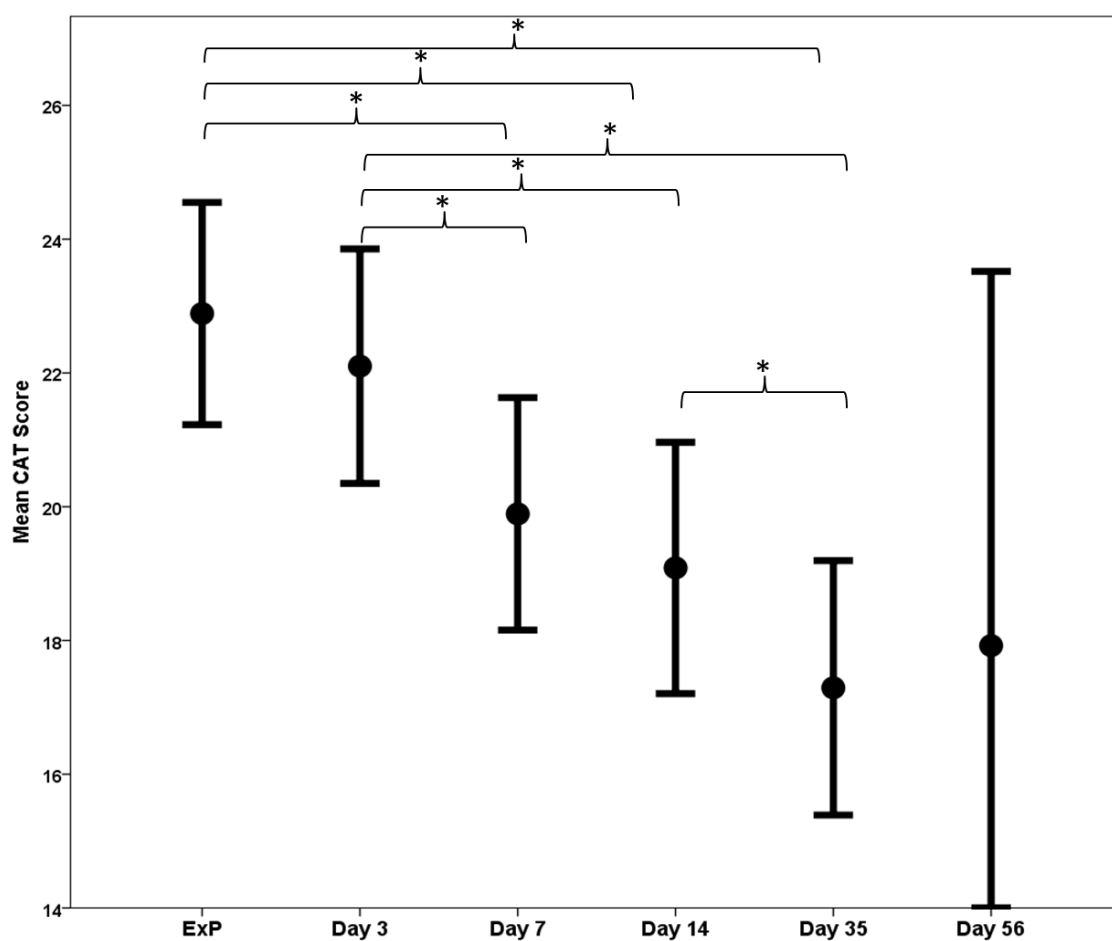


Figure 7.7: Change in CAT scores during COPD exacerbation and recovery period. Data shown as mean (\pm 95% CI).

When exploring differences in CAT scores with or without HRV infection, it was found that samples positive for HRV (n=107) had a significantly higher mean CAT score than those samples in which HRV was not detected (n=281) (Figure 7.8). In HRV-positive samples, the mean CAT score was 22.12(\pm 0.8) which was significantly higher than the mean CAT score in those samples found to be negative for HRV, 19.95(\pm 0.5) (p=0.018). Samples at all time point were included in the analysis.

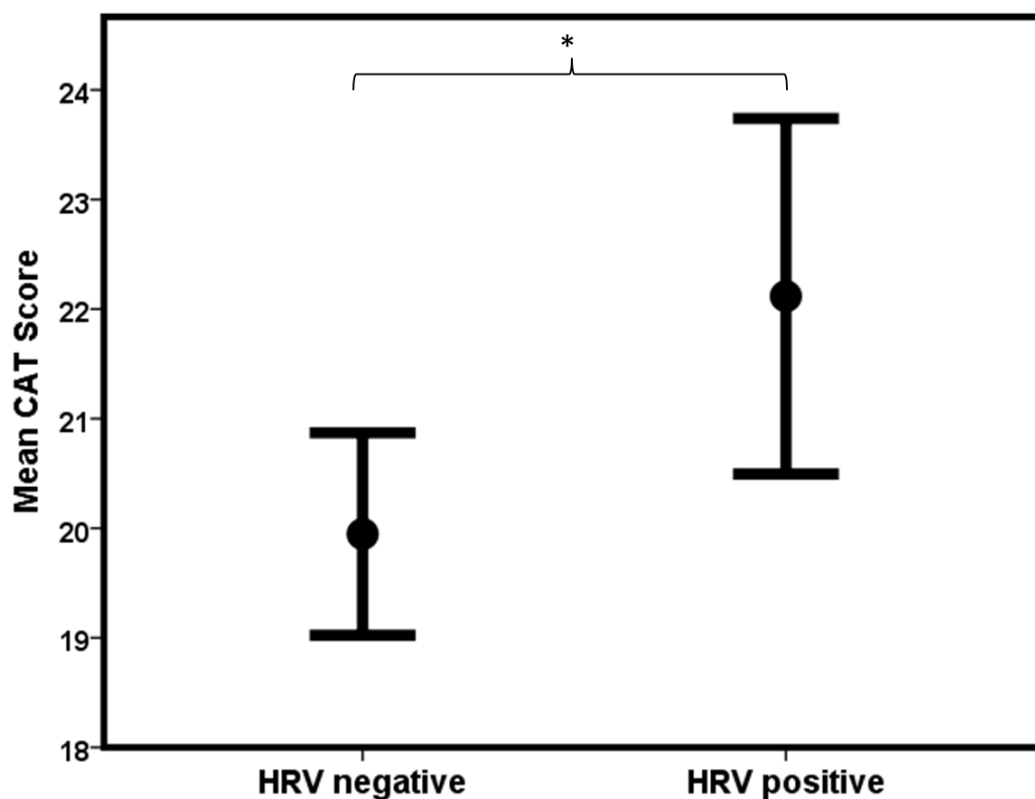


Figure 7.8: Comparison of mean CAT scores in samples positive for HRV (n=107) and samples negative for HRV (n=281). All time-points included; *p=0.018.

The mean CAT scores were highest at ExP and decreased over the time-course exacerbation recovery, as did HRV load. There were significant falls from exacerbation presentation (ExP) to Day 7 for both variables (both $p<0.015$) (Figure 7.9).

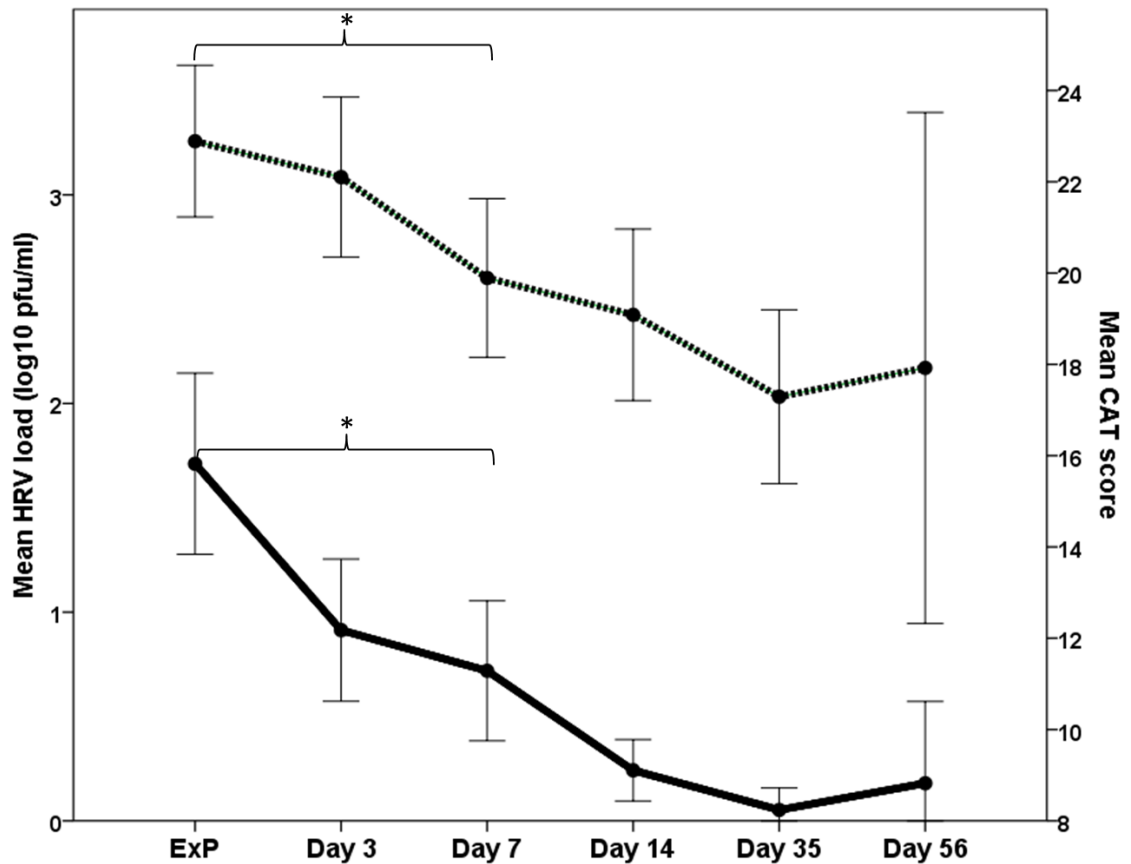


Figure 7.9: Change in CAT scores and HRV load over the time-course of COPD exacerbation and recovery; * $p\leq 0.015$.

There was a significant relationship between HRV load and CAT scores with CAT scores increasing as HRV load increased; $r=0.149$, $p=0.003$. All time-points included (Figure 7.10).

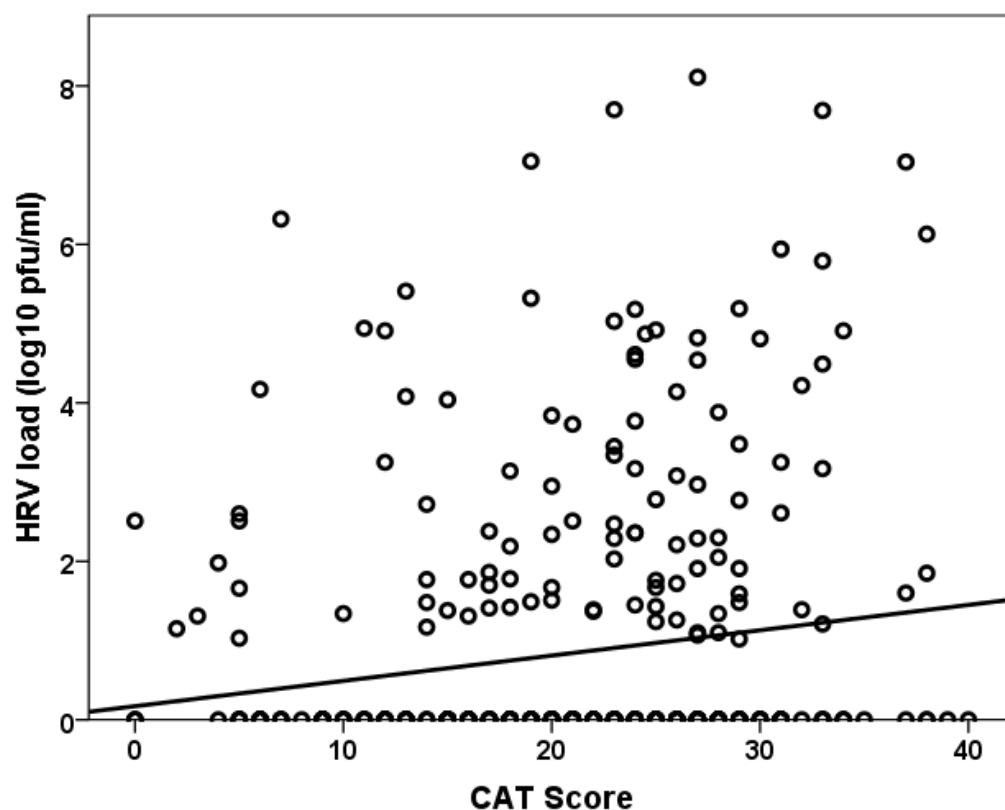


Figure 7.10: Relationship of CAT scores and HRV load over the time-course of COPD exacerbation and recovery. All time-points included; $r=0.149$, $p=0.003$.

The CAT is designed to assess and quantify the impact of COPD symptoms on patient health status and like the EXACT, exploring any differences in CAT scores between patients who reported URT symptoms and those that did not, requires investigation.

In samples associated with cold symptoms at the time of sampling (n=144), the mean(\pm SEM) CAT score was found to be 22.8(\pm 0.67) which was significantly higher in those not associated with a cold (n=244), 19.3(\pm 0.50); $p < 0.001$. All time-points included (Figure 7.11). There was no significant difference between the mean CAT scores in samples associated with sore throats (n=58) and those that were not (n=330); $p = 0.201$.

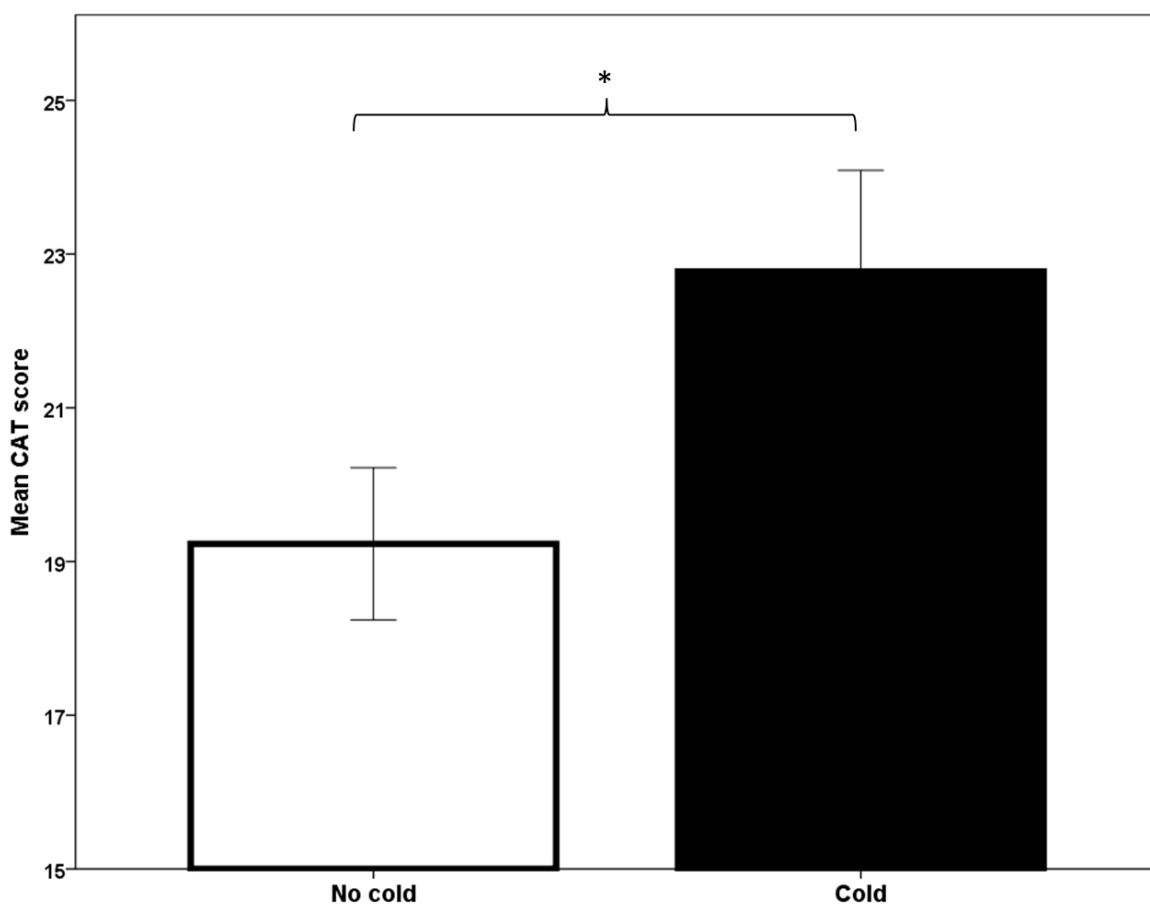


Figure 7.11: Comparison of CAT scores at all time-points in samples associated with cold symptoms at the time of sampling (n=144) and those that were not (n=244); All time-points included * $p < 0.001$.

7.6 Characteristics of patients used for exacerbation frequency analysis

For 73 patients there was data on the number of exacerbations they had per year retrospectively. The 73 samples taken from 73 patients were analysed between May 2010 and December 2011 as part of the sample collection performed in the London COPD cohort. The baseline clinical characteristics for these patients are shown in Table 7.3.

FEV ₁ , litres	1.28 (\pm 0.5)
FVC, litres	2.25 (\pm 0.9)
FEV ₁ /FVC,	0.49 (\pm 0.15)
Predicted FEV ₁ , %	48.9 (\pm 19.6)
Current Smoker, %	32
Age, years	72.4 (\pm 7.6)
Male gender, %	63

Table 7.3: Baseline clinical characteristics of 73 patients in the London COPD Cohort, who participated in a study of HRV infection and exacerbation frequency. Data are presented as mean (\pm SD) or percentage (%).

7.7 Results – exacerbation frequency

Of these 73 exacerbations, patients positive for HRV (n=43) during their first sampled exacerbation, had a significantly higher exacerbation frequency than those patients in whom HRV was not detected (n=30). The median (IQR) number of exacerbations per year in those with HRV infection was 3.01 (2.02-5.28) compared to 2.51 (1.88-3.51) in those without HRV detected (p=0.040) (Figure 7.12).

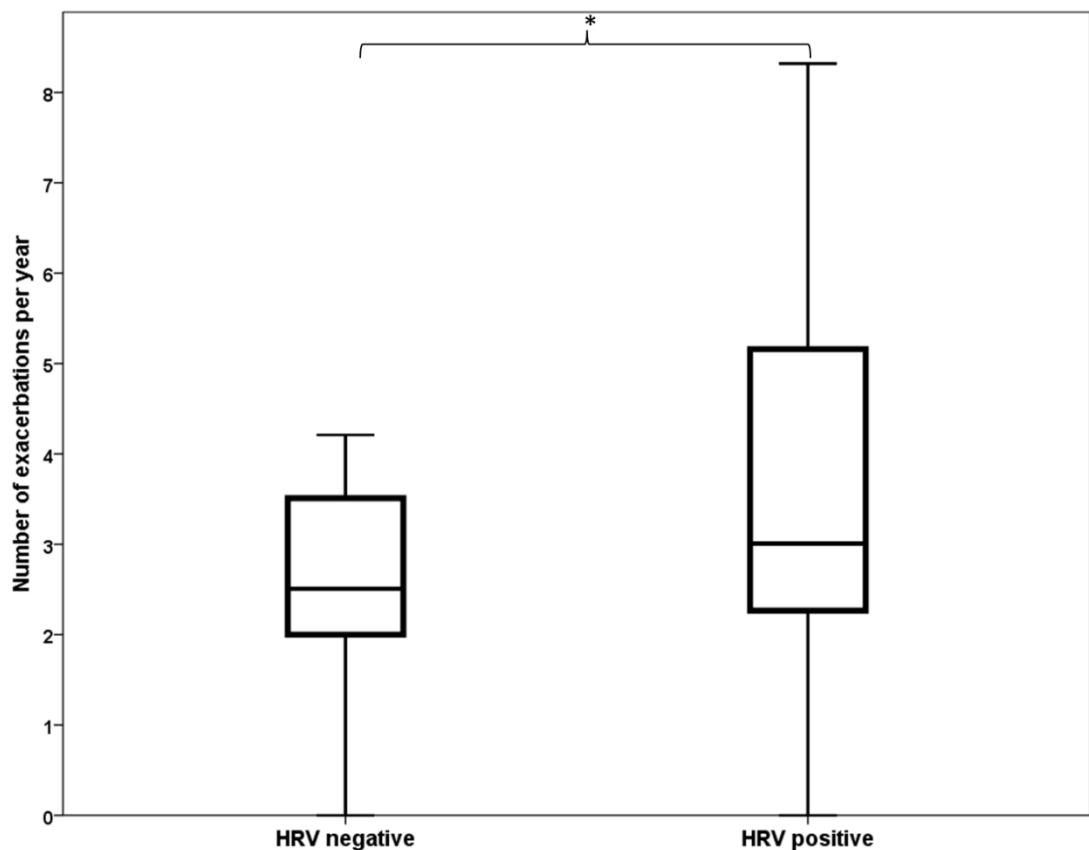


Figure 7.12: Comparison of the number of exacerbations per year in patients with HRV (n=43) and those without HRV (n=30) at exacerbation. *p=0.04. Data shown as median (IQR).

In addition, HRV load at exacerbation was significantly related to the annual rate of exacerbations ($r=0.265$, $p=0.024$) (Figure 7.13).

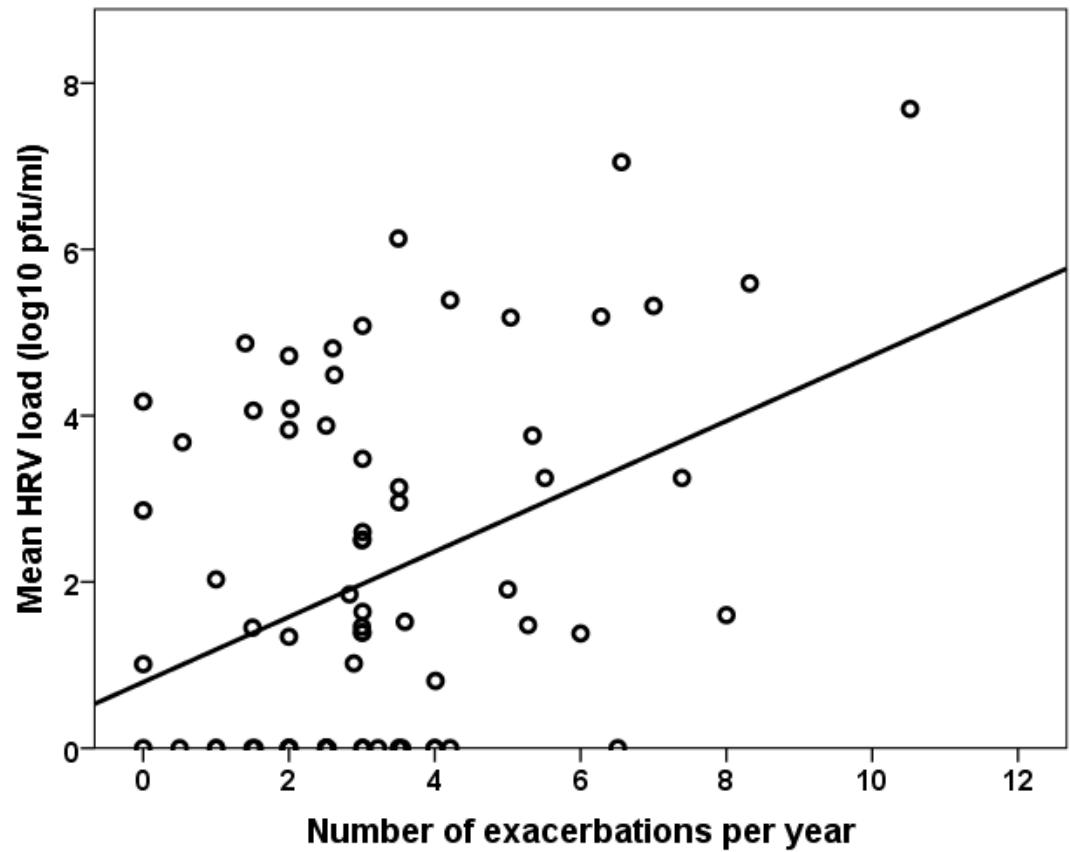


Figure 7.13: Relationship of exacerbation frequency and HRV load over the time-course of COPD exacerbation and recovery; $r=0.024$, $p=0.265$.

7.8 Discussion

7.8.1 EXACT questionnaire

The EXACT questionnaire is a patient reported outcome measure that allows the standardisation of COPD exacerbation severity (Leidy et al., 2011). The EXACT is a validated patient reported outcome tool that has been used to enhance the detection of exacerbation events in clinical trials such as the FORWARD trial that aimed to reduce exacerbations with a combination inhaler (Singh et al., 2013) and a trial exploring the effectiveness of alerting COPD patients when they are at a high risk of exacerbation (Halpin et al., 2011). Previous studies have also investigated the effectiveness of the EXACT at predicting exacerbations by recording EXACT scores over the time-course of an exacerbation and recovery period. It was found that EXACT scores accurately reflected exacerbation recovery with scores increasing significantly at COPD exacerbation and decreasing during recovery (Leidy et al., 2011; Mackay et al., 2014). Furthermore, EXACT scores measured over seven days were shown to be higher at each time point in exacerbating patients compared to stable patients (Leidy et al., 2011). The current study shows EXACT scores to be highest at exacerbation and decreasing during the recovery period, consistent with previous studies. For the first time, the current study explored the association of EXACT scores over the entire time-course of COPD exacerbations with HRV infection. The results showed that COPD patients with HRV infection during the time-

course of an exacerbation and recovery had higher EXACT scores than those without HRV. Furthermore there was a significant relationship between HRV load and EXACT scores over the time-course with EXACT scores increasing as HRV load increased. These findings suggest that exacerbations associated with HRV infection are more severe in terms of greater burden of symptoms. Both EXACT scores and HRV load were highest at exacerbation presentation and decreased during recovery with significant decreases in both from exacerbation presentation to Day 3 post-exacerbation. This implies that EXACT scores and HRV load follow a similar pattern over COPD exacerbation time-course suggesting the EXACT questionnaire could be used to track symptomatic recovery in HRV-infected patients which in turn may affect the timing of therapy in COPD exacerbations.

Interestingly, EXACT scores were found to be higher in the presence of upper respiratory (URT) symptoms such as cold symptoms and sore throats. The EXACT questionnaire is comprised of questions that largely focus on symptoms of the lower airway such as breathlessness, cough and sputum, chest symptoms and difficulty bringing up sputum as opposed to questions about URT symptoms such as sore throats or nasal symptoms. However it seems from the results reported in this study that there is an association between URT symptoms and EXACT scores with EXACT scores being significantly higher in samples associated with URT symptoms compared to those without symptoms. In Chapter 5 it was reported that URT symptoms may be used to indicate the presence of HRV infection as HRV prevalence and load was shown to be higher in the presence of URT

symptoms. This raises questions about the potential ability of the EXACT questionnaire, in conjunction with URT symptoms, to indicate the presence of HRV infection at COPD exacerbation and determine the severity of these HRV-associated exacerbations. Personal communication from research fellows in the London COPD cohort suggests that patients often have difficulty completing the EXACT questionnaire due to its length and complexity and prefer to use the CAT questionnaire. This must be taken into consideration when preparing to use the EXACT as it appears too complex for clinical use, however may be of potential benefit in further HRV research studies.

7.8.2 CAT questionnaire

Similar results were found when exploring the association of HRV prevalence and load with CAT scores. The CAT is designed to assess and quantify the impact of COPD symptoms on patient's health status (Jones et al., 2009). It provides clinicians and patients with a simple and reliable measure of COPD-related health status allowing the assessment and long-term follow-up of individual patients (Jones et al., 2009). The CAT has been shown to be associated with changes in systemic inflammation following COPD exacerbations and is responsive to treatments given (Tu et al., 2014). It has also been shown to improve in response to pulmonary rehabilitation programmes and is able to differentiate between categories of response (Dodd et al., 2011). CAT scores have been shown to be significantly higher at COPD exacerbation compared to the stable state (Jones

et al., 2011; Mackay et al., 2012) and can distinguish between patients of different degrees of COPD severity (Jones et al., 2011). Scores have been shown to reflect recovery after exacerbations of COPD with the time taken for scores to return to baseline being significantly related to recovery time as judged by symptom diary cards (Mackay et al., 2012). Results from the current study are consistent with these results and found CAT scores to be highest at exacerbation presentation before decreasing during the recovery period. For the first time, the association of CAT scores and HRV infection during COPD exacerbations and recovery has been explored where CAT scores were found to be significantly higher in HRV-positive samples compared to those without HRV infection, throughout the time-course. Furthermore, both HRV load and CAT scores were shown to be highest at exacerbation presentation and decrease significantly by Day 7 post-exacerbation and were found to be weakly but significantly related during the time-course with higher HRV loads correlating with higher CAT scores. Results from the current study also indicate that CAT scores are higher in the presence of cold symptoms. As discussed in relation to EXACT scores, it was shown in Chapter 5 that HRV infection is associated with URT symptoms. Therefore together, URT symptoms along with EXACT and CAT scores could be used as potential indicators of HRV infection at exacerbation. Moreover, the EXACT and CAT scores could be used to assess efficacy of novel therapies by determining exacerbation severity in future trials of antiviral therapy at COPD exacerbation and during recovery. Additionally the results from this study reinforce the importance of developing antiviral therapies for HRV to allow appropriate treatments to be given to COPD patients

and should encourage further research into the development of treatment for HRV infection.

7.8.3 Exacerbation frequency

Exacerbation frequency has been shown to be an important phenotype in COPD (Donaldson et al., 2013). Some patients with COPD are particularly susceptible to having exacerbations and are termed “frequent exacerbators” (Hurst et al., 2010). It has been shown that patients are able to shift from being frequent exacerbators to infrequent exacerbators or vice versa and therefore all patients are at some risk of becoming a frequent exacerbators during the course of COPD (Donaldson et al., 2013). In 2002, Donaldson and colleagues reported that exacerbation frequency is an important determinant of lung function in COPD and showed that patients with frequent exacerbations had a significantly faster decline in FEV₁ and peak expiratory flow (PEF) compared to infrequent exacerbators (Donaldson et al., 2002). It was also reported that patients with frequent exacerbations were often admitted to hospital with longer length of stay (Donaldson et al., 2002). A study by Patel and colleagues explored exacerbation frequency and lower airway bacterial colonisation in stable COPD patients and found that lower airway bacterial colonisation in stable COPD modulates the frequency of COPD exacerbations as colonisation with a pathogen was associated with an increased exacerbation frequency (Patel et al., 2002). In 2005 it was shown that patients with the

frequent exacerbator phenotype are more likely to develop colds compared to non-frequent exacerbators (Hurst et al., 2005a). In the study, frequent exacerbators developed significantly more colds than infrequent exacerbators but it was concluded that the likelihood of developing an exacerbation during a cold was not affected by exacerbation frequency but that frequent exacerbators were more susceptible to acquiring a cold (Hurst et al., 2005). Various mechanisms were suggested to explain why frequent exacerbators have an increased frequency of colds; frequent exacerbators being intrinsically more susceptible to viral infection, the host-viral interaction in these patients is such that an exacerbation is more likely, or that patients with frequent colds may be coming into contact with more viruses in the community (Hurst et al., 2005). It is also known that HRV is able to infect the lower airways as well as the upper airways (Seemungal et al., 2000). The data suggests frequent exacerbators are predisposed to the acquisition of colds rather than greater risk of exacerbation during a cold.

This work by Hurst and colleagues focussed on cold symptoms and exacerbation frequency but did not explore HRV infection specifically, in these patients. For the first time, the current study explored the association of HRV prevalence and load with exacerbation frequency in COPD patients during the entire time-course of an exacerbation and recovery period. The results showed that COPD patients with HRV infection at exacerbation had a significantly higher exacerbation frequency than those without HRV. The results also demonstrated a weak but significant correlation between exacerbation

frequency and HRV load showing that HRV load increased as exacerbation frequency increased. This suggests that frequent exacerbators are more susceptible to respiratory viral infection or are less able to prevent viral replication. A proposed mechanism of increased viral susceptibility is the modulation of intracellular adhesion molecule (ICAM)-1 on respiratory epithelial cells. ICAM-1 functions as the receptor for major group HRV (Staunton et al., 1989). It has been shown that ICAM-1 is upregulated in the bronchial mucosa of patients with chronic bronchitis (Di Stefano et al., 1994), which may lead to increased HRV infection in these patients. Furthermore, frequent exacerbators have been shown to have increased airway inflammation in the stable state (Bhowmik et al., 2000) and that COPD is associated with increased nasal inflammation (Hurst et al., 2005; Vachier et al., 2004). Inflammation leads to the upregulation of ICAM-1 (Staunton et al., 1989) which in turn suggests that the increases ICAM-1 and therefore HRV infection, due to inflammation in the upper airway of frequent exacerbators, could account for these findings. The potential mechanisms suggested by Hurst and colleagues to explain why frequent exacerbators have an increased frequency of colds (Hurst et al., 2005a) could be applied to these results to explain increased susceptibility to HRV infection seen in frequent exacerbators.

This increase in susceptibility to HRV infection in frequent exacerbators may have important consequences in terms of treatment of exacerbations and should promote further study into the development of an HRV-specific antiviral or vaccine. These results

emphasise the importance of treating HRV infections with the aim of reducing exacerbation frequency and preventing exacerbation recurrence in these patients.

7.9 Conclusion

For the first time, this study has explored the association of HRV infection with EXACT scores, CAT scores and exacerbation frequency. COPD patients with HRV infection have higher EXACT and CAT scores throughout the time-course of an exacerbation and recovery compared to those without HRV. Furthermore, as HRV load increases, both EXACT and CAT scores also increase suggesting both questionnaires could be used in future trials of antiviral therapy and also to track symptom recovery.

This study also showed an association between HRV infection and exacerbation frequency. COPD patients with HRV infection had higher exacerbation frequency than those without HRV and HRV load at exacerbation was greater in patients with a higher exacerbation frequency. These results emphasise the importance of the development of an HRV-specific antiviral to prevent HRV-associated exacerbations and with the aim of reducing exacerbation frequency.

CHAPTER 8. Respiratory syncytial virus in stable COPD and at exacerbation

8.1 Introduction

This pilot study aimed to investigate the prevalence and semi-quantitative load of respiratory syncytial virus (RSV) in COPD patients in the stable state and at naturally occurring exacerbation. This study also explored the association of RSV infection with upper and lower respiratory tract symptoms.

As discussed in previous chapters, HRV has been shown to be the most commonly detected respiratory virus at COPD exacerbation (Seemungal et al., 2001) with RSV being the second most commonly detected (Seemungal et al., 2001). At exacerbation, RSV has been detected at various prevalence rates by different groups, which reflects the uncertainty around the role of RSV in COPD exacerbations. Seemungal and colleagues found RSV to be present in 14.2% of exacerbation samples (Seemungal et al., 2001), and Rohde reported RSV in 22% (Rohde et al., 2003), whereas Falsey and colleagues only detected RSV in 7.2% of exacerbation samples (Falsey et al., 2006). RSV commonly infects children and infants (Lanari et al., 2014; Martinelli et al., 2014) however it has also been shown to cause substantial disease in many adult populations such as the elderly, healthy younger populations, immunocompromised individuals and subjects with COPD (Walsh, 2011). A study exploring RSV infection in adults found that 9-11.4% of COPD patients with RSV-associated exacerbations required hospitalisation and 13% of these needed intensive care treatment (Falsey, 2005). Another study identifying risk factors for RSV illness in

COPD patients found that 11.1% patients were infected with RSV with almost half needing medical attention (Mehta et al., 2013). Furthermore it was shown that congestive heart failure and exposure to children were risk factors for symptomatic RSV illness (Mehta et al., 2013). Persistent RSV infection in COPD patients has been associated with accelerated FEV₁ decline (Matsumoto and Inoue, 2014; Wilkinson et al., 2006), higher levels of inflammatory markers (Seemungal et al., 2001) and worse lung function (Wilkinson et al., 2006a). Interestingly, the mortality rates of RSV-associated COPD exacerbations are greater than those for influenza despite influenza being a more commonly recognised virus (Falsey, 2005) although this may be due to the effect of the influenza vaccination given to COPD patients.

RSV has been detected in stable COPD as well as at exacerbation leading to some controversy about RSV persistence verses intermittent acute infection. Some groups suggest that RSV persists in the lung long-term whereas others believe it plays a greater role in acute infection. A study by Borg and colleagues in 2003 reported a similar prevalence of RSV in both stable COPD and at exacerbation (27.9% vs 28.3%, respectively) (Borg et al., 2003) however Seemungal and colleagues reported the prevalence of RSV to be higher in stable COPD (23.5%) compared to exacerbation (14.2%) (Seemungal et al., 2001). Conversely, Papi and colleagues found the prevalence of RSV to be higher at COPD exacerbation compared to the stable state and did not find RSV to persist in COPD patients (Papi et al., 2006). A study exploring the contribution of RSV on cigarette smoke-

induced airway inflammation and lung tissue destruction showed that RSV does not just colonise the lungs but is an important aetiological factor in driving inflammation, apoptosis and tissue destruction in smoke-exposed mice (Foronjy et al., 2014). Other studies report the feasibility of RSV to persist in the airways through evasion and escape from the host's immune system, along with impaired immunity found in COPD patients (Sikkel et al., 2008). One study detected RSV using PCR in nasopharyngeal samples from 28.3% COPD patients that were hospitalised for reasons other than acute exacerbation. However, the RSV loads in these patients were found to be lower than the RSV loads found in children with acute RSV infection (Borg et al., 2003).

RSV is an enveloped RNA virus in the *Paramyxoviridae* family (Walsh, 2011). RSV is classified into two distinct groups, RSV-A and RSV-B based on antigenic and genetic variability (Anderson et al., 1985) however it is not clear whether infection by these two strains affects COPD differently, or whether there is a difference in the prevalence and/or load of the two groups at different disease states (Mufson et al., 1985). A study in which mice were infected with two different strains of RSV-A at similar viral titres, found that strain-specific variations in cytokine expression, goblet cell hyperplasia MUC5AC expression and airway hyper-reactivity occurred which suggests that RSV strain differences may be an important component of RSV disease severity (Lukacs et al., 2006). Studies involving humans however reported no differences in viral effects of infection with different RSV strains. In a study of elderly patients and high-risk adults, RSV-A was

responsible for 45% of infection and RSV-B for 55% (Falsey, 2005). To date, there is little information about differences in the prevalence of the two strains in stable COPD and at exacerbation. There is also no data at present on the changes in load between these two disease states for either individual strain.

This pilot study investigated RSV prevalence and semi-quantitative load as a whole in COPD patients in the stable state and at naturally occurring COPD exacerbation, but also as individual serotypes, RSV-A and RSV-B, at these two time-points. Furthermore, this study aimed to explore the association of RSV infection with upper and lower respiratory tract symptoms at exacerbation. The semi-quantitative PCR methodology for RSV is described in *section 2.5.6*.

8.2 Characteristics of patients in unpaired analysis

A total of 416 stable state samples and 119 exacerbation samples from 142 COPD patients were collected for RSV analysis between January 2009 and January 2013 as part of the sample collection performed in the London COPD cohort. The baseline clinical characteristics for the 142 patients are shown in Table 8.1 below.

FEV ₁ , litres	1.12 (\pm 0.5)
FVC, litres	2.28 (\pm 0.9)
FEV ₁ /FVC, %	47.1 (\pm 14.9)
Predicted FEV ₁ , %	44.9 (\pm 20.3)
Current Smoker, %	32
Age, years	68.4 (\pm 7.8)
Male gender, %	64

Table 8.1: Baseline clinical characteristics of 142 patients in the London COPD Cohort, who participated in study of RSV presence at COPD exacerbation and in the stable state in unpaired samples. Data are presented as mean (\pm SD) or percentage (%).

8.3 Results - unpaired analysis

8.3.1 RSV prevalence in unpaired analysis

From a total of 416 stable state samples, 91 (21.9%) had RSV detected. Of 119 exacerbation samples, 18 (15.1%) were positive for RSV which was not significantly different to the RSV prevalence in the stable state ($p=0.092$) (Figure 8.1). Only 2 of the 18 samples positive for RSV at exacerbation were also positive for RSV in the stable state.

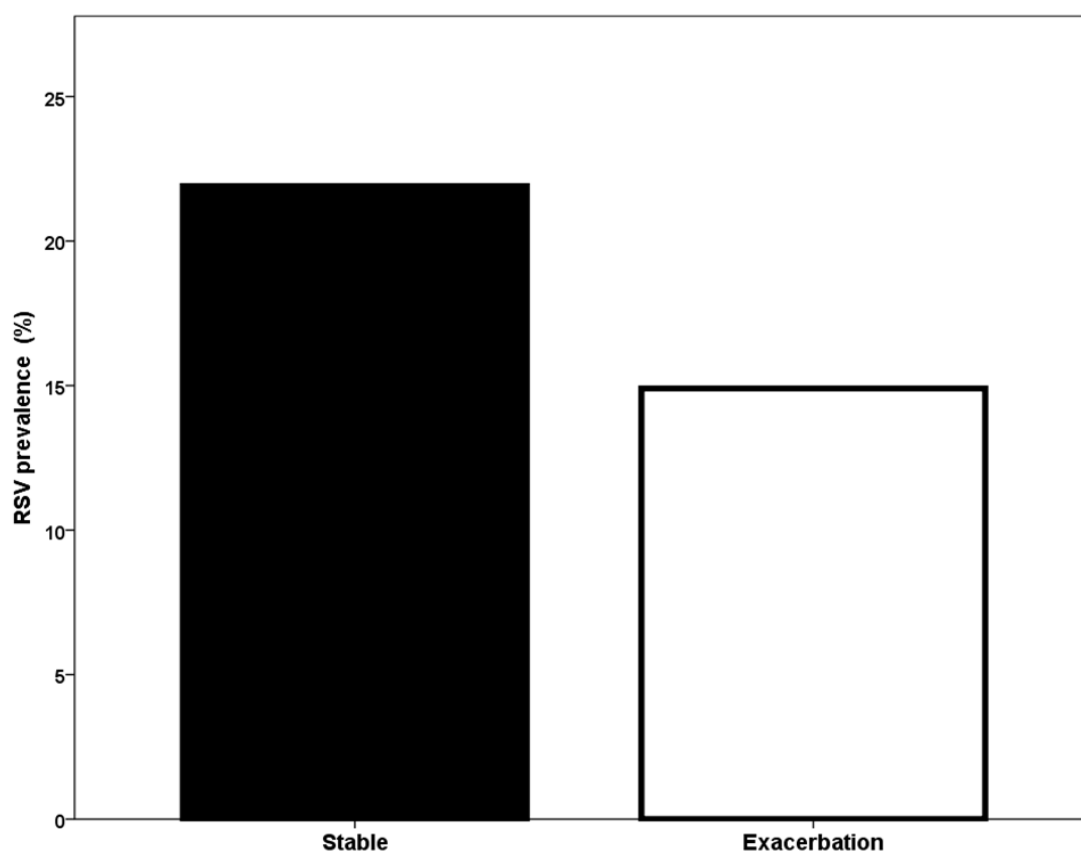


Figure 8.1: Comparison of RSV prevalence in the stable state (n=416) and at COPD exacerbation (n=119) in unpaired samples.

From a total of 416 stable state samples, 90 (21.6%) had RSV-A detected. Of 119 exacerbation samples, 15 (12.6%) were positive for RSV-A which was significantly lower than in the stable state ($p=0.023$) (Figure 8.2).

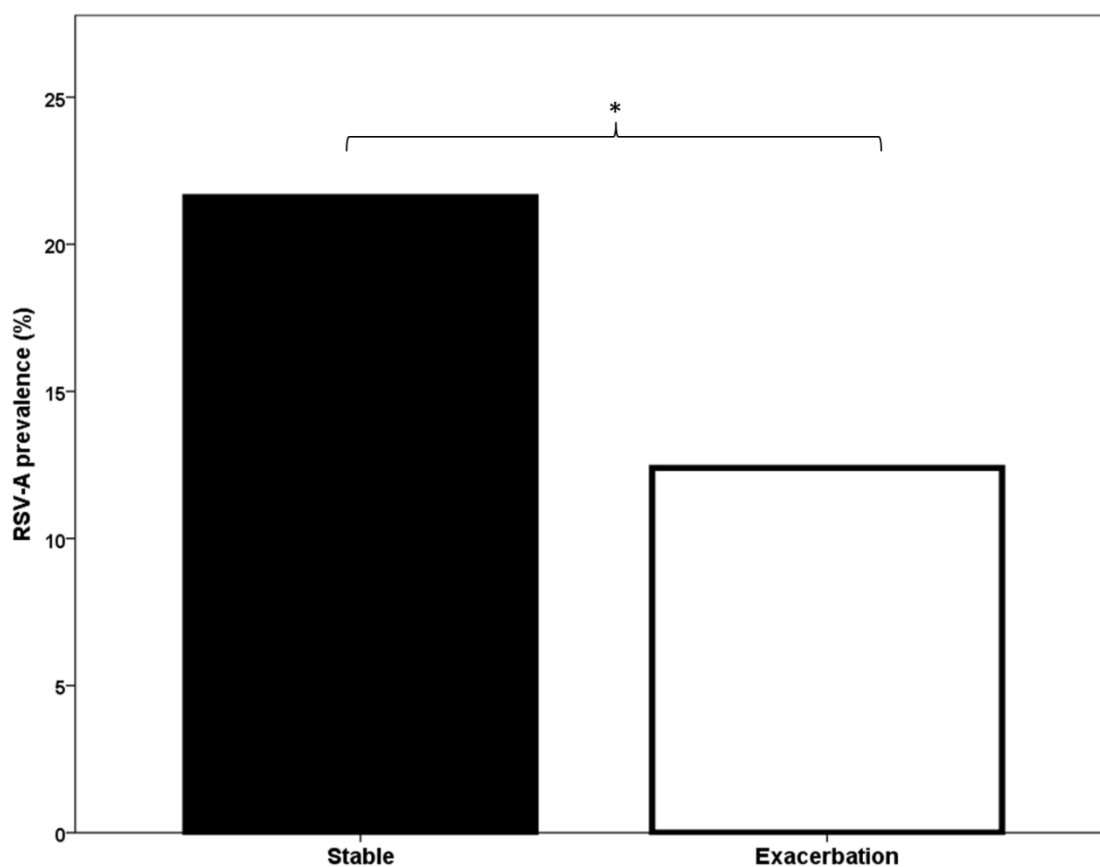


Figure 8.2: Comparison of RSV-A prevalence in the stable state (n=416) and at COPD exacerbation (n=119) in unpaired samples; * $p=0.023$.

From a total of 416 stable state samples, 2 (0.5%) had RSV-B detected. Of 119 exacerbation samples, 3 (2.5%) were positive for RSV-B which was significantly higher than in the stable state ($p=0.044$) (Figure 8.3).

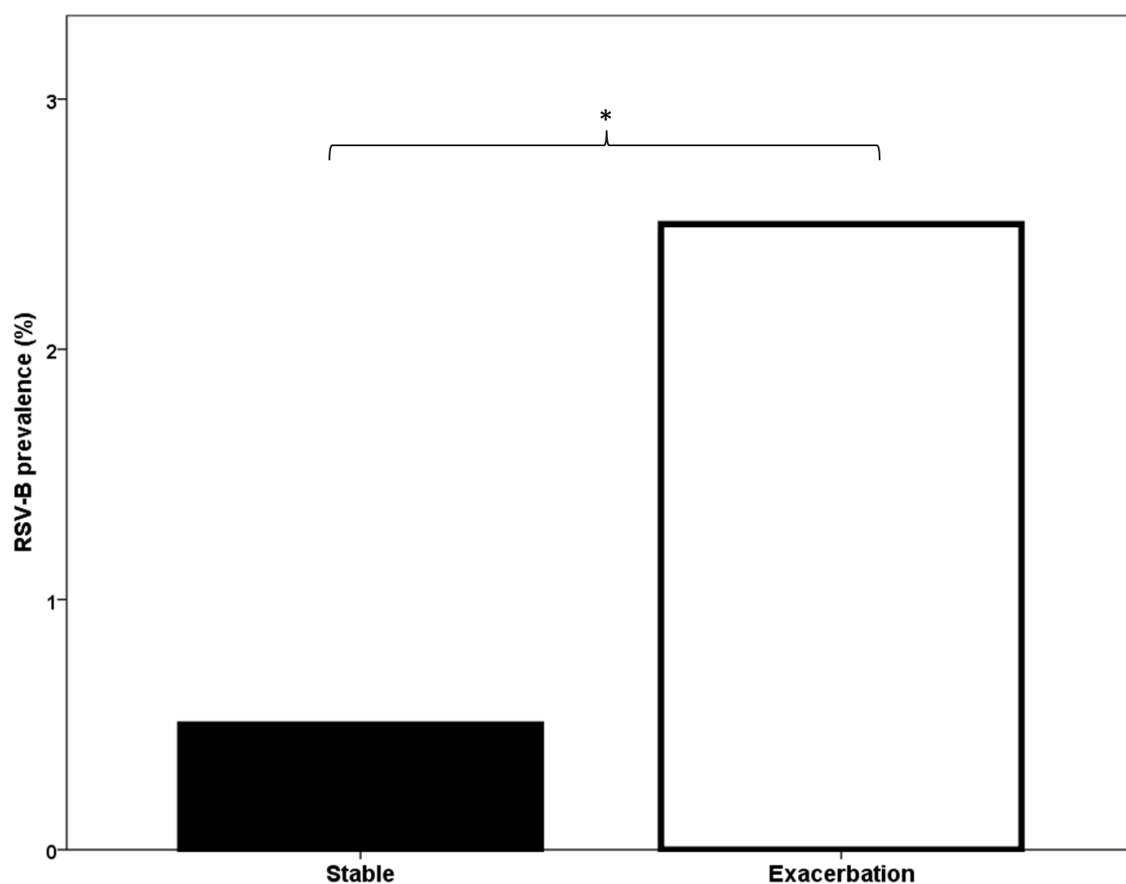


Figure 8.3: Comparison of RSV-B prevalence in the stable state (n=416) and at COPD exacerbation (n=119) in unpaired samples; * $p=0.044$.

8.3.2 RSV and upper respiratory tract symptoms

Of the 119 exacerbation samples, 87 had recorded symptom data; 76 of these negative for RSV and 11 positive for RSV.

Of the 11 samples positive for RSV, 3 (27.3%) were associated with cold symptoms at exacerbation. Of the 76 samples negative for RSV, 28 (36.8%) were associated with cold symptoms. There was no significant difference between the prevalence of cold symptoms between these two groups; $p=0.536$ (Figure 8.4).

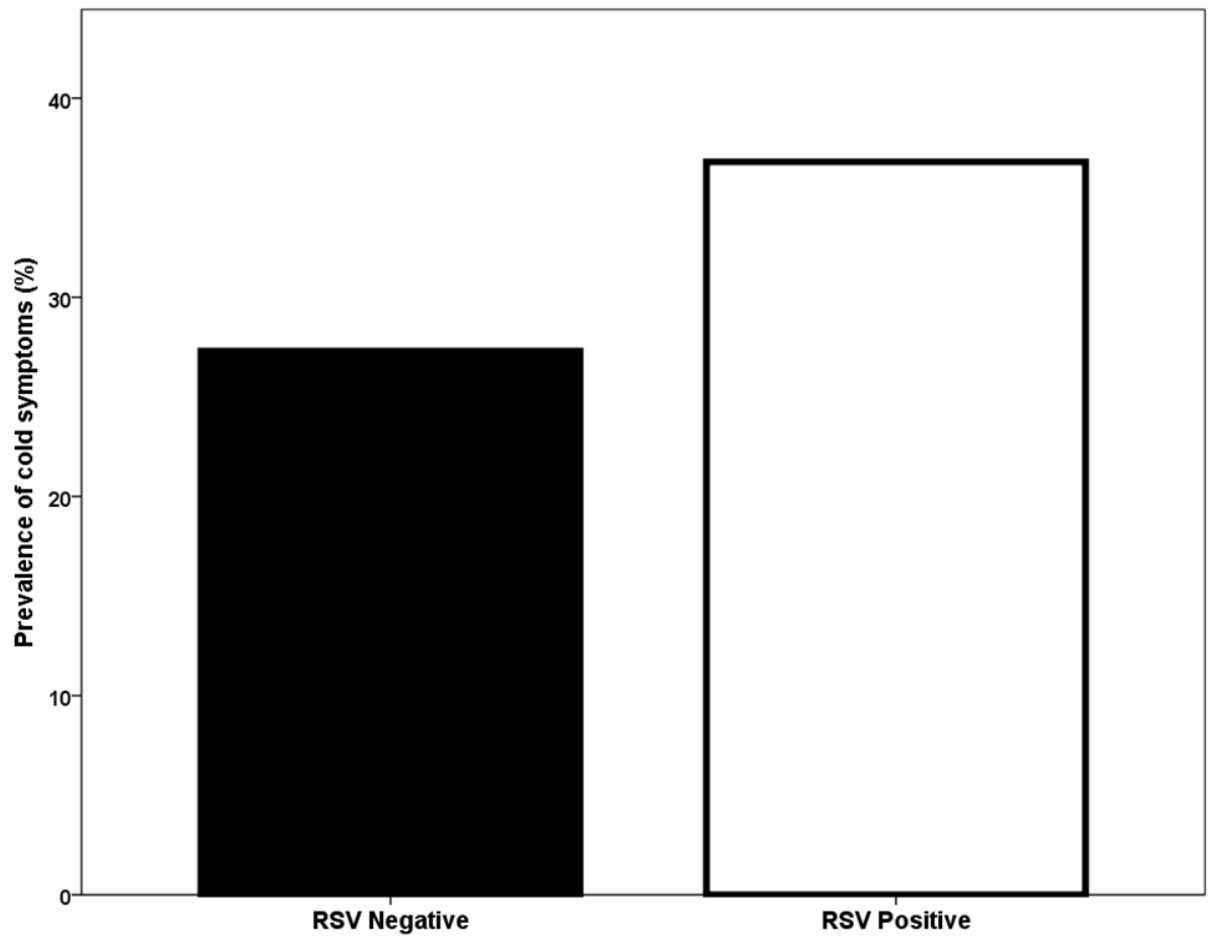


Figure 8.4: Comparison of the prevalence of cold symptoms at exacerbation in RSV positive samples (n=11) and RSV negative samples (n=76).

Of the 11 samples positive for RSV, 0 (0%) were associated with sore throat symptoms at exacerbation. Of the 76 samples negative for RSV, 8 (10.5%) were associated with sore throat symptoms. There was no significant difference between the prevalence of sore throats between these two groups; $p=0.259$ (Figure 8.5).

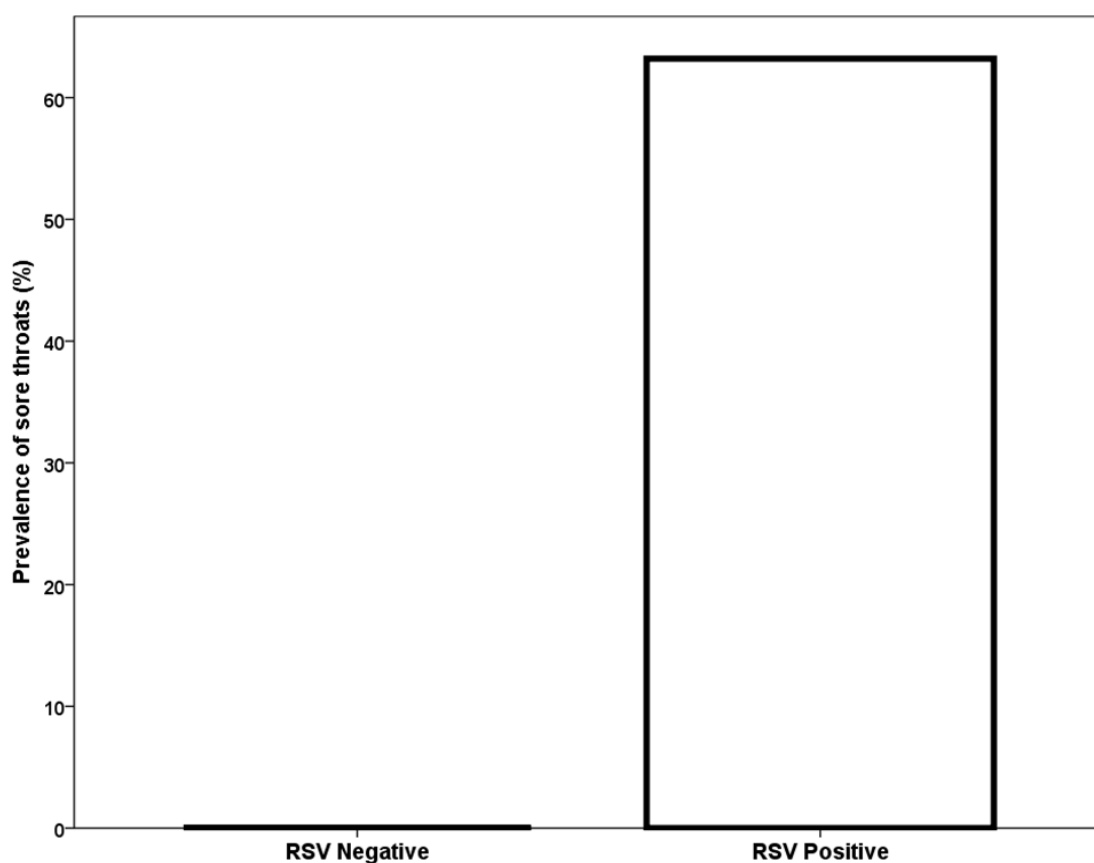


Figure 8.5: Comparison of the prevalence of sore throats at exacerbation in RSV positive samples (n=11) and RSV negative samples (n=76).

Of the 87 exacerbation samples that had recorded symptom data, 28 (32.3%) were associated with cold symptoms only, 3 (3.4%) were associated with sore throats only and 3 (3.4%) were associated with both symptoms. The prevalence of cold symptoms only in this sample set, was significantly higher than for sore throats only ($p<0.001$) or for both symptoms ($p<0.001$) (Figure 8.6).

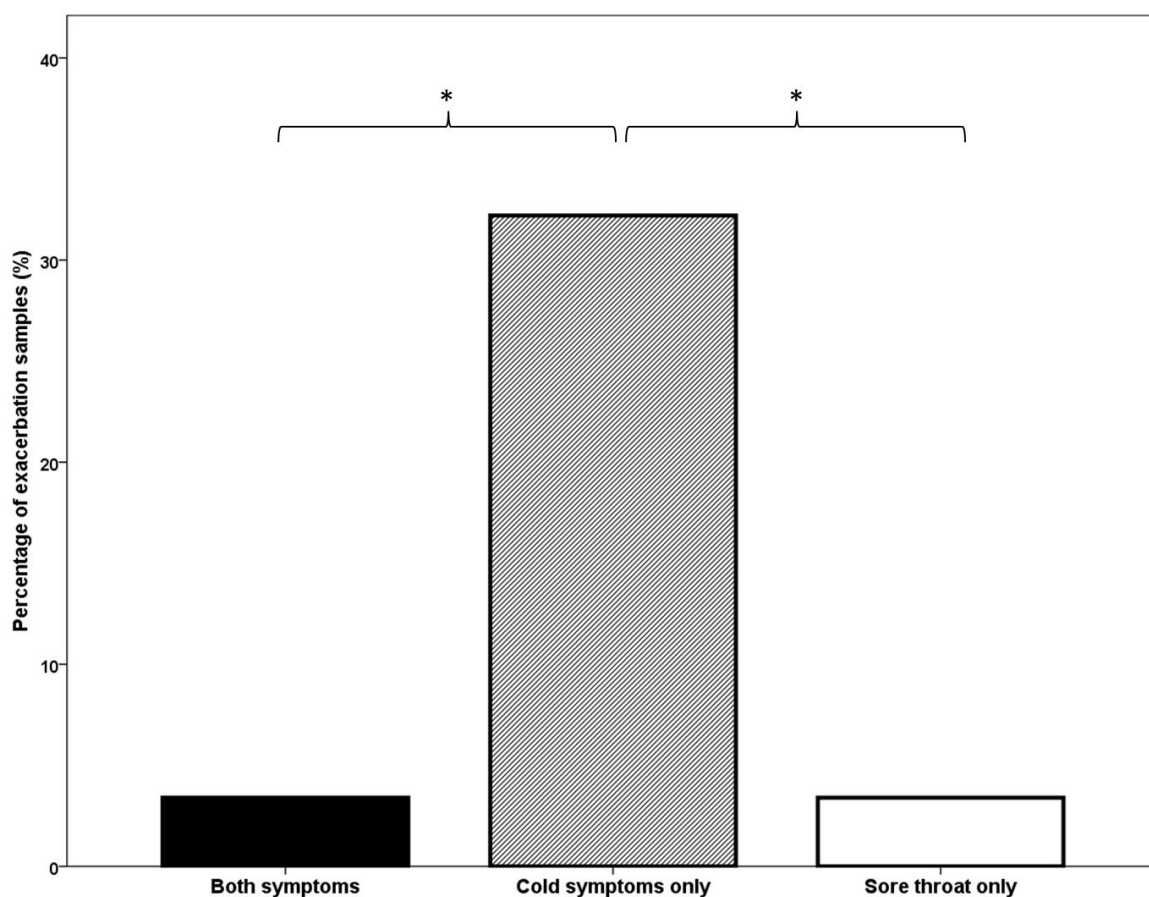


Figure 8.6: Comparison of the percentage of samples associated with cold symptoms only (n=28), sore throats only (n=3) or both symptoms (n=3) at exacerbation; * $p<0.001$.

The prevalence of RSV in samples associated with cold symptoms only, was found to be 3/28 (10.7%). There were no positive RSV samples associated with sore throats only, or both symptoms.

8.3.3 RSV and lower respiratory tract symptoms

Of the 11 samples positive for RSV, 7 (63.6%) were associated with an increase in sputum volume at exacerbation. Of the 76 samples negative for RSV, 43 (56.6%) were associated with an increase in sputum volume. There was no significant difference between the prevalence of increased sputum volume between these two groups; $p=0.658$ (Figure 8.7).

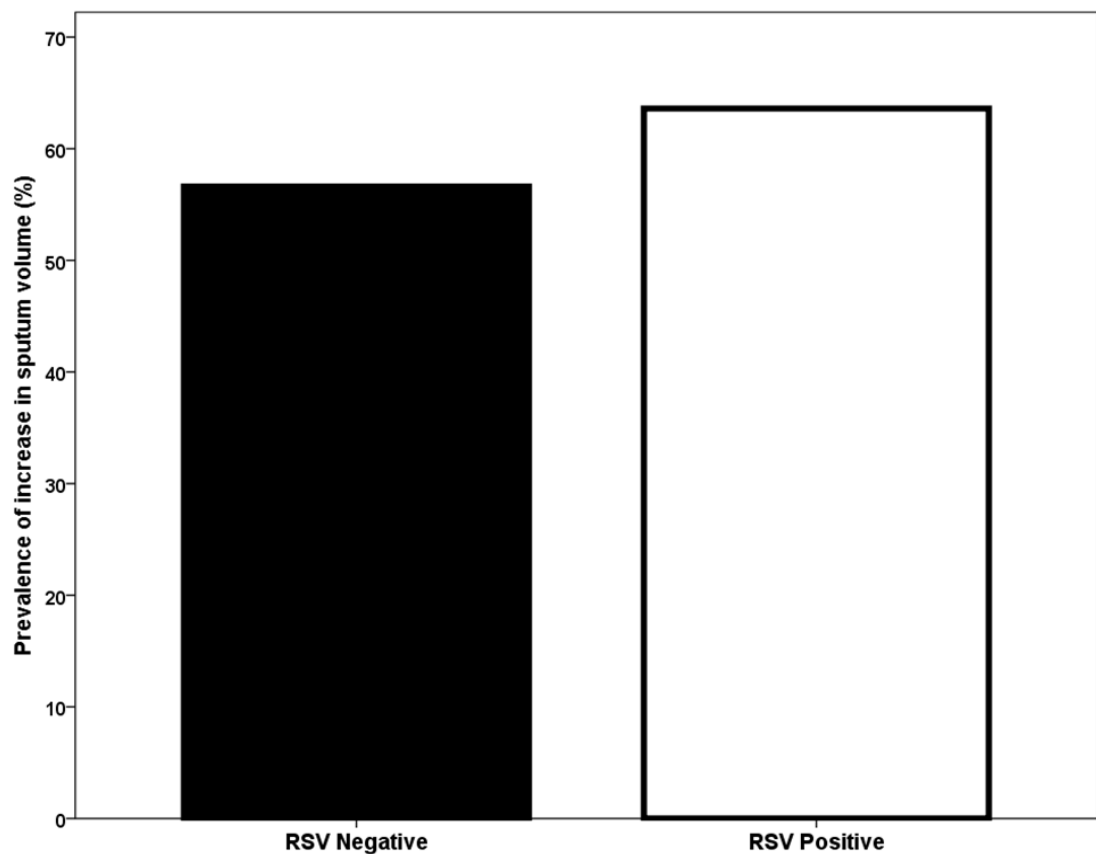


Figure 8.7: Comparison of the prevalence of increased sputum volume between samples negative for RSV (n=76) and samples positive for RSV (n=11).

Of the 11 samples positive for RSV, 5 (45.5%) were associated with an increase in sputum purulence at exacerbation. Of the 76 samples negative for RSV, 38 (50.0%) were associated with an increase in sputum purulence. There was no significant difference between the prevalence of increased sputum purulence between these two groups; $p=0.778$ (Figure 8.8).

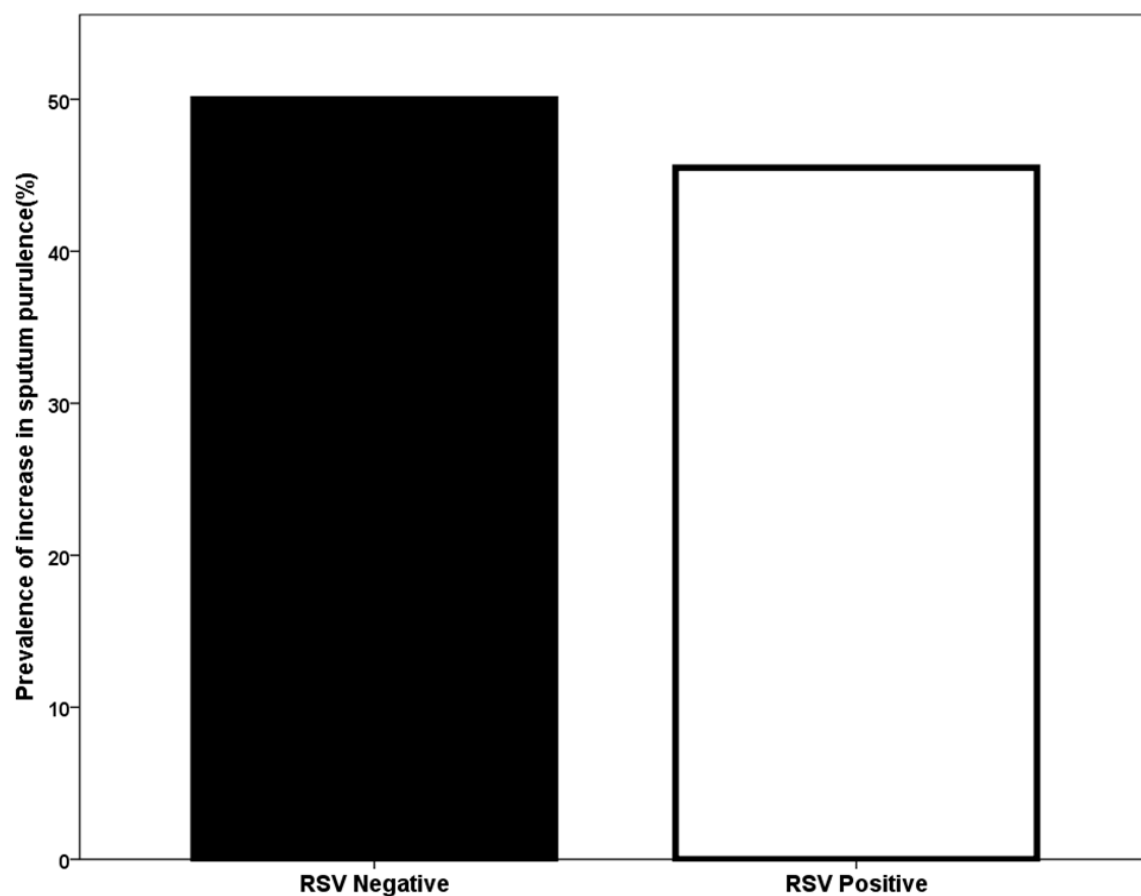


Figure 8.8: Comparison of the prevalence of increased sputum purulence between samples negative for RSV (n=76) and samples positive for RSV (n=11).

There were insufficient sample numbers to analyse sputum volume and sputum purulence changes in subjects with RSV-A or RSV-B, specifically.

8.3.4 RSV and CRP levels

CRP data was available for 357 of 416 stable state samples.

In stable state samples positive for RSV (n=75), the median (IQR) CRP value was found to be 3 (1-6) pg/ml which was not significantly different to the median level of CRP in stable state samples negative for RSV (n=282) which was 3 (1-7) pg/ml; $p=0.685$ (Figure 8.9).

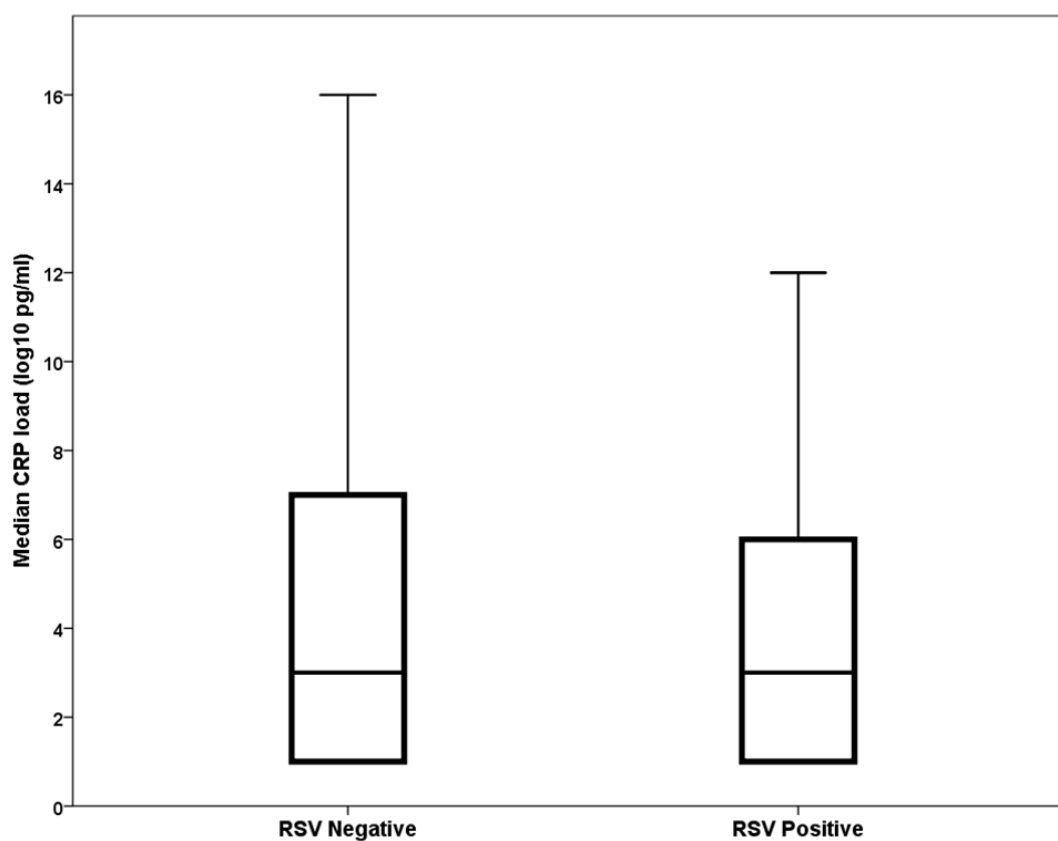


Figure 8.9: Comparison of the CRP levels in the stable state in RSV negative samples (n=282) and RSV positive samples (n=75).

In stable state samples positive for RSV-A (n=74), the median (IQR) CRP value was found to be 3 (1-6.25) pg/ml which was not significantly different to the median level of CRP in stable state samples negative for RSV-A (n=282) which was 3 (1-7) pg/ml; $p=0.682$ (Figure 8.10).

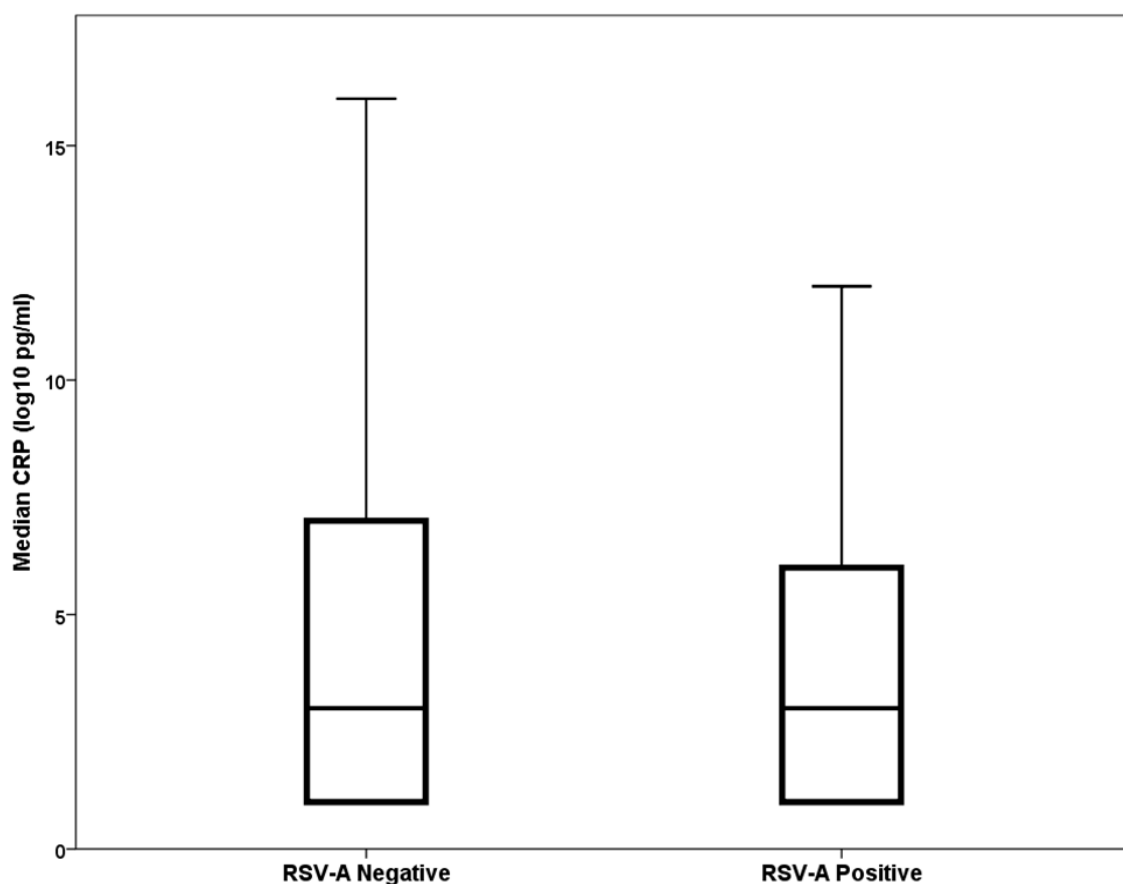


Figure 8.10: Comparison of the CRP levels in the stable state in RSV-A negative samples (n=283) and RSV-A positive samples (n=74).

There was insufficient data for CRP levels to be analysed in RSV-B positive samples.

8.3.5 RSV semi-quantitative load in unpaired analysis

The RSV load was measured semi-quantitatively which used the detection threshold (Ct value) to compare the amount of RSV between samples allowing relative quantification rather than absolute quantification of viral loads to be determined. A lower Ct value indicates a higher viral load, as less PCR cycles are required to amplify the cDNA within a sample. More specifically, a decrease in Ct value by one, equates to a 2-fold increase in viral load.

In unpaired samples that were positive for RSV-A (n=90 in the stable state and n=15 at exacerbation), it was identified that the median (IQR) detection threshold was 38.40 (37.77-38.73) in the stable state which was 2-fold lower than 37.28 (33.26-38.04) at exacerbation (Figure 8.11).

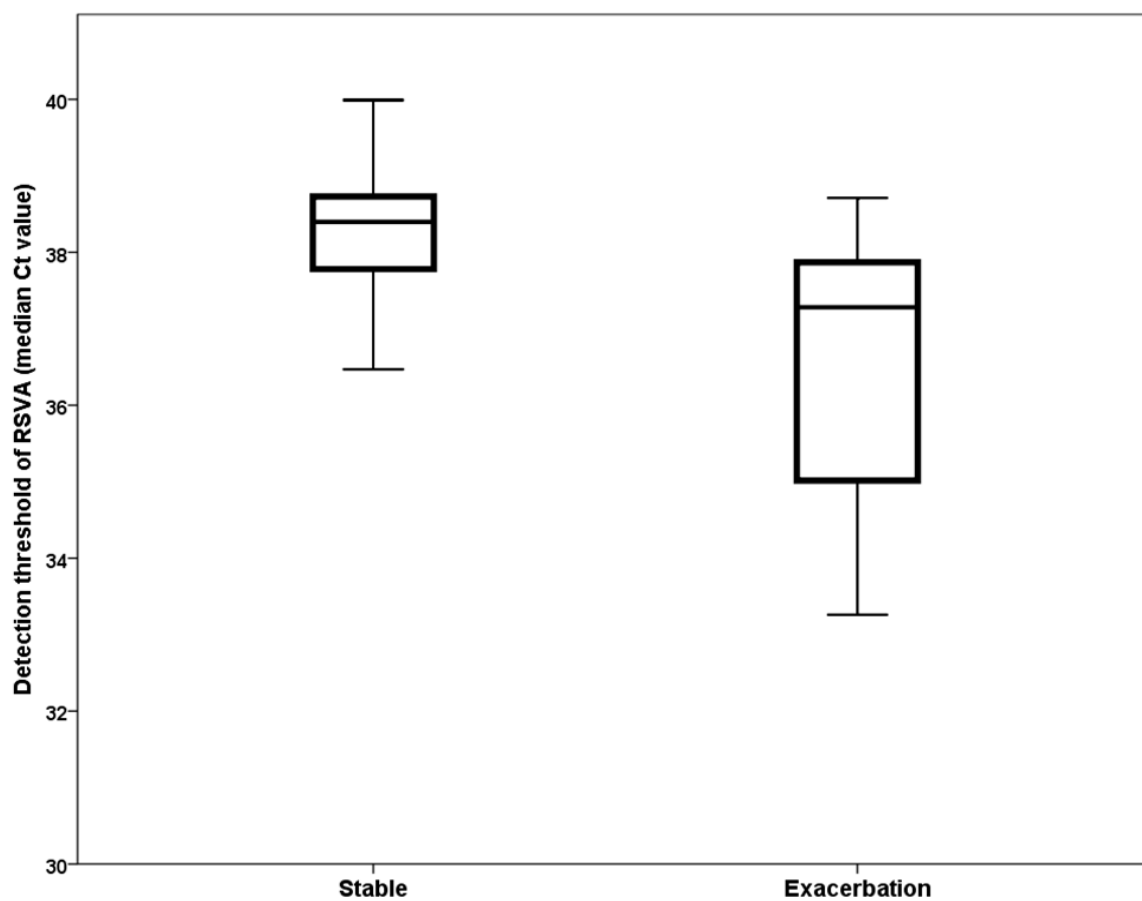


Figure 8.11: Comparison of RSV-A detection threshold in the stable state (n=90) and at COPD exacerbation (n=15) in unpaired samples. Note that Ct value is inversely correlated with RSV load i.e. higher RSV loads will have a lower detection threshold.

In unpaired samples that were positive for RSV-B (n=2 in the stable state and n=3 at exacerbation), it was identified that the median (IQR) detection threshold was 38.96 (38.61-39.31) in the stable state which was approximately 16-fold lower than 35.19 (26.22-39.30) at exacerbation (Figure 8.12).

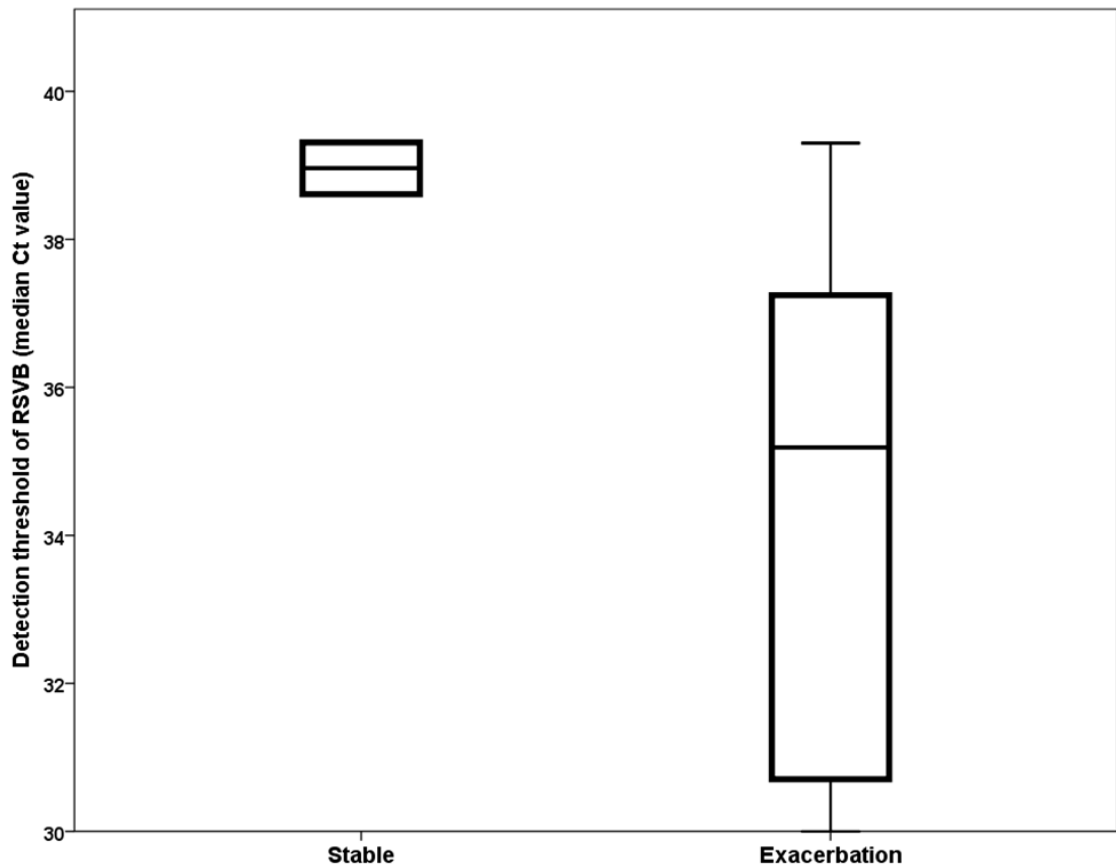


Figure 8.12: Comparison of RSV-B detection threshold in the stable state (n=2) and at COPD exacerbation (n=3) in unpaired samples. Note that Ct value is inversely correlated with RSV load i.e. higher RSV loads will have a lower detection threshold.

8.1 Characteristics of patients in paired analysis

A sub-analysis of 32 COPD patients who had paired stable and exacerbation state sputum samples was performed to reduce any bias that may occur when different patients are used to reflect changes in RSV prevalence and load at different COPD states. Pairs of sputum samples, one taken in the stable state and one at exacerbation were analysed. A total of 45 stable state samples and 45 exacerbation samples from the 32 patients were analysed between April 2009 and December 2012. This sub-analysis involved stable state samples being obtained less than 365 days prior to an exacerbation. The baseline clinical characteristics of the 32 patients included are shown in Table 8.2 below.

FEV ₁ , litres	1.12 (\pm 0.5)
FVC, litres	2.27 (\pm 0.9)
FEV ₁ /FVC, %	46.9 (\pm 14.9)
Predicted FEV ₁ , %	44.9 (\pm 20.3)
Current Smoker, %	32
Age, years	78.6 (\pm 8.0)
Male gender, %	64

Table 8.2: Baseline clinical characteristics of 32 patients in the London COPD Cohort, who participated in study of RSV presence at COPD exacerbation and in the stable state in paired samples. Data are presented as mean (\pm SD) or percentage (%).

8.2 Results – paired analysis

8.2.1 RSV prevalence in paired analysis

From a total of 45 stable state sputum samples, it was found that 8 (17.8%) were positive for RSV. Of 45 exacerbation samples, 3 (6.7%) showed such presence which was not significantly different to RSV prevalence in the stable state ($p=0.108$) (Figure 8.13).

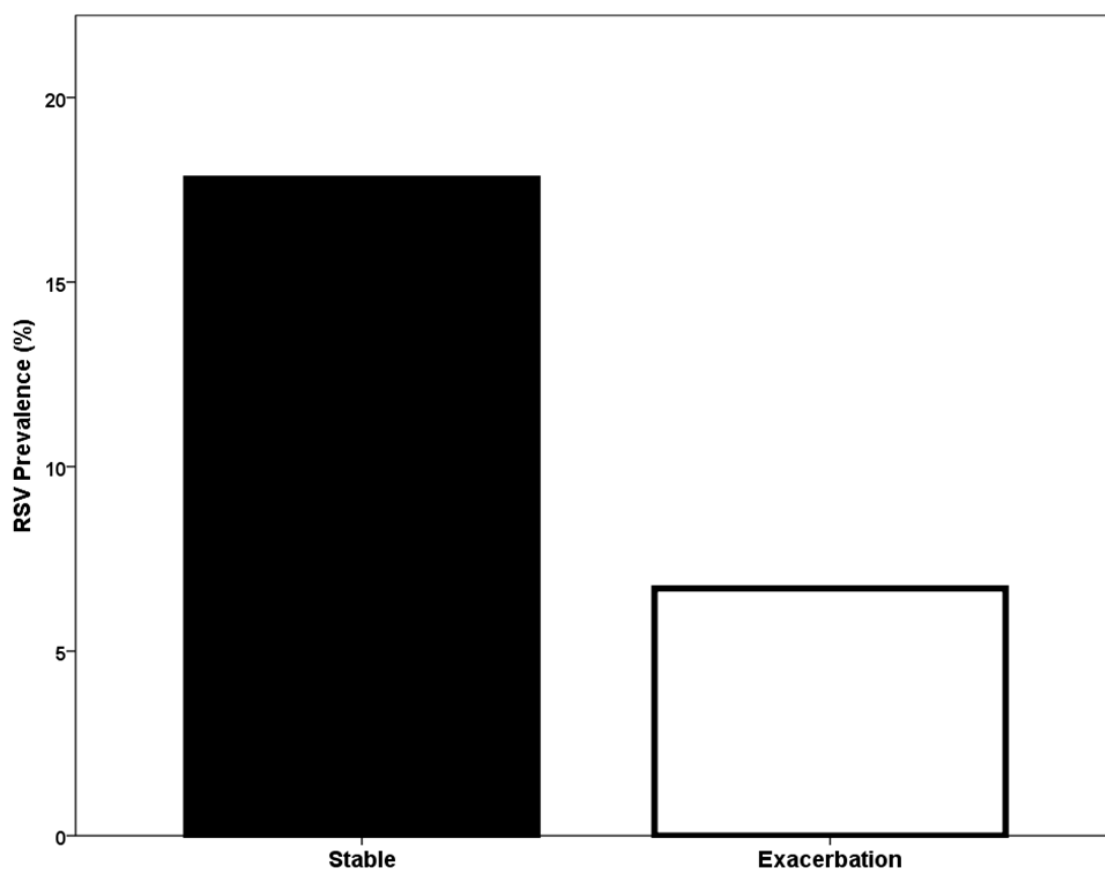


Figure 8.13: Comparison of RSV prevalence in the stable state ($n=45$) and at COPD exacerbation ($n=45$) in paired samples.

From a total of 45 stable state sputum samples, it was also found that 8 (17.8%) were positive for RSV-A. Of 45 exacerbation samples, 3 (6.7%) showed such presence which was not significantly different to RSV-A prevalence in the stable state ($p=0.108$) (Figure 8.14). RSV-B was not detected in any of the paired stable state or exacerbation samples, reflecting the low prevalence in both disease states.

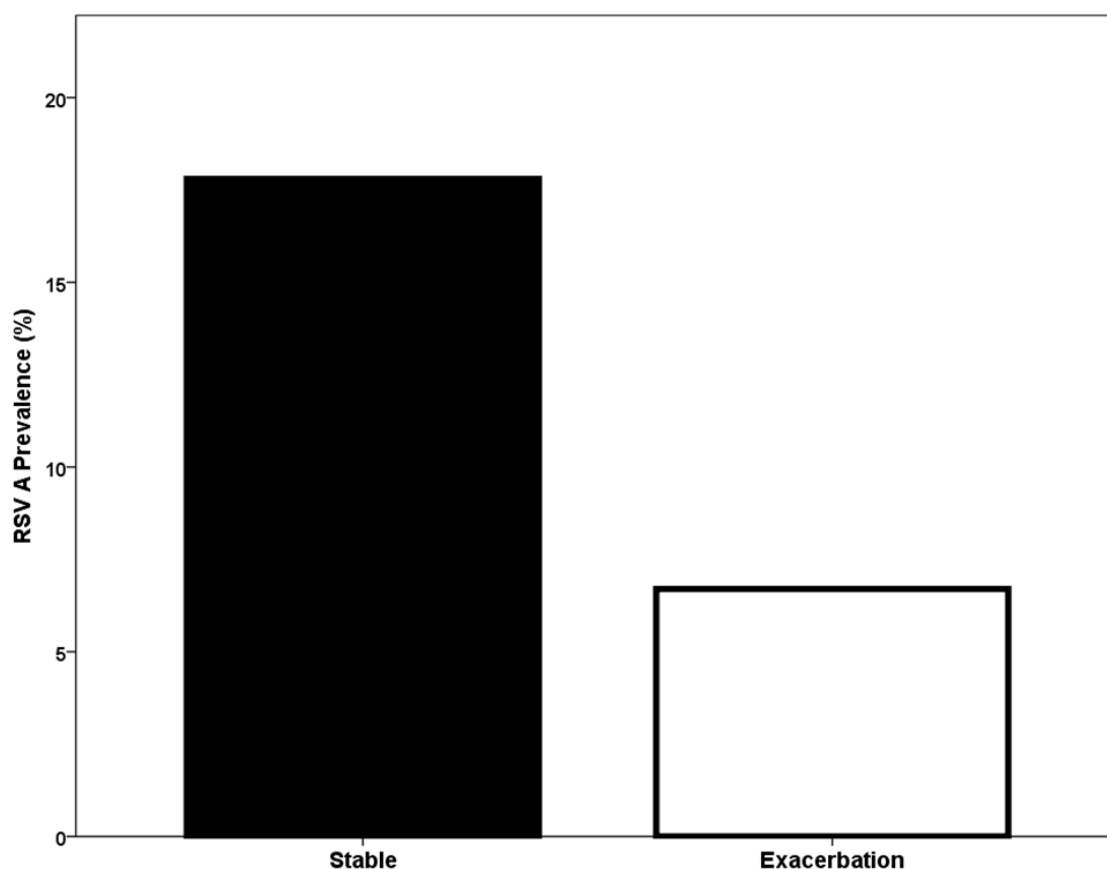


Figure 8.14: Comparison of RSV-A prevalence in the stable state (n=45) and at COPD exacerbation (n=45) in paired samples.

8.2.2 RSV semi-quantitative load in paired samples

As with the unpaired analysis, the RSV load was measured semi-quantitatively which used the detection threshold (Ct value) to compare the RSV levels between samples allowing relative quantification rather than absolute quantification of viral loads to be determined. The lower the Ct value, the higher the viral load as less cycles are required to amplify the cDNA within a sample.

In paired samples that were positive for RSV-A (n=8 in the stable state and n=3 at exacerbation), it was identified that the median (IQR) detection threshold was 38.12 (37.12-38.66) in the stable state which did not change at exacerbation, 38.08 (37.62-38.11) (Figure 8.15). RSV-B was not detected in any of the paired stable state or exacerbation samples.

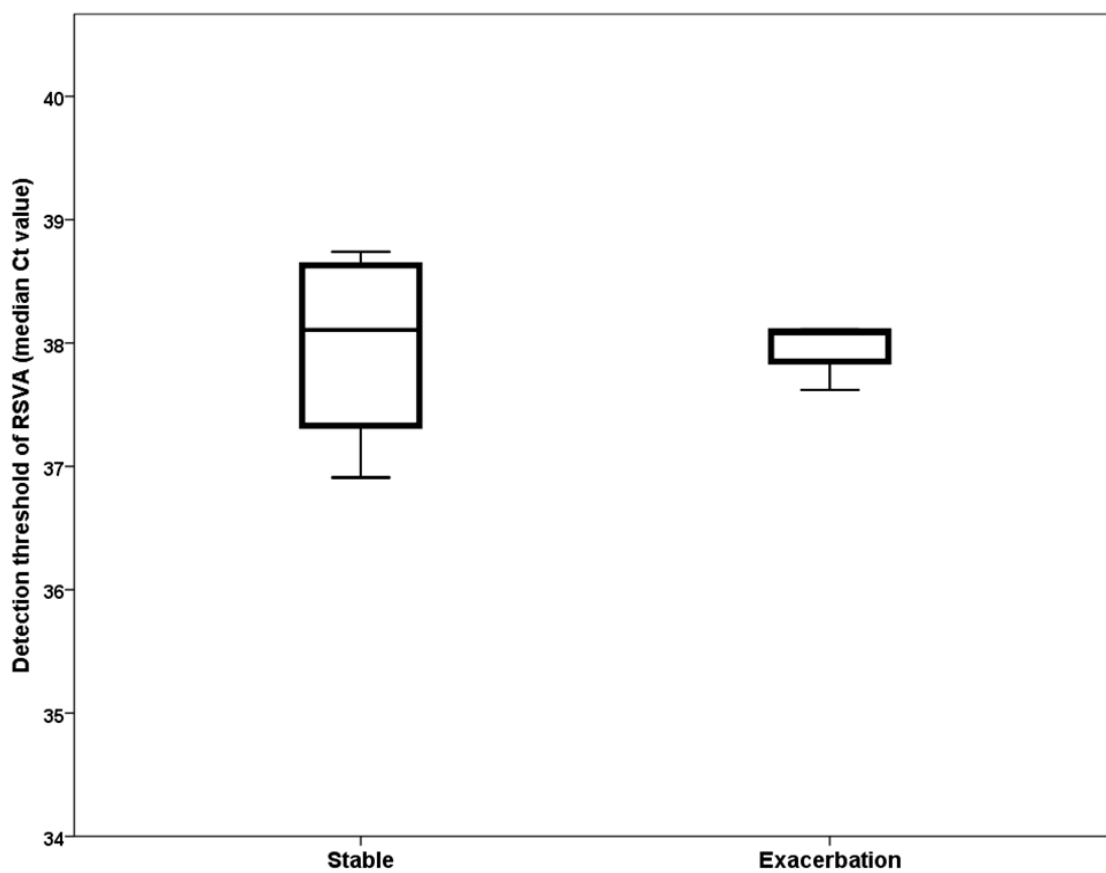


Figure 8.15: Comparison of RSV-A detection threshold in the stable state (n=8) and at COPD exacerbation (n=3) in paired samples. Note that Ct value is inversely correlated with RSV load i.e. higher RSV loads will have a lower detection threshold.

8.3 Discussion

This pilot study investigated RSV infection in stable COPD and at exacerbation, exploring the prevalence and semi-quantitative load of RSV as a whole, and also of RSV-A and RSV-B as separate subtypes of RSV. It also considered the association of RSV infection with upper and lower respiratory tract symptoms, and with levels of C-reactive protein (CRP).

Controversy still remains about the role of RSV in COPD; some studies have shown RSV to persist in the airways (Borg et al., 2003; Wilkinson et al., 2006) while others consider it to play a larger role in acute infection (Papi et al., 2006). RSV has been shown to be the most commonly detected virus in stable COPD (Seemungal et al., 2001), however it has also been shown to be the second most commonly detected virus at COPD exacerbation (Seemungal et al., 2001).

A study by Seemungal and colleagues in 2001 detected RSV in 23.5% of stable state samples which was higher than the 14.2% detected at COPD exacerbation and there was no association of exacerbation samples with higher RSV loads compared to the stable state suggesting low-grade asymptomatic infection (Seemungal et al., 2001). Furthermore, a study by Wilkinson and colleagues found positive RSV detection in 32.8% of stable state samples from COPD patients using PCR and sequencing techniques (Wilkinson et al., 2006). In 2003, Borg and colleagues used quantitative PCR to detect and quantify RSV in

nasopharyngeal aspirates from infected children, and in nasal lavage and sputum samples from COPD patients, in both the stable state and at exacerbation. RSV detection rates were found to be similar in both disease states, 27.9% vs 28.3% respectively. Using viral load measurements it was shown that the RSV load was nearly 2000-fold higher in the infected children compared to the COPD patients in both the stable state and at exacerbation, which again, suggests low-grade, and potentially persistent, RSV infection in patients with COPD (Borg et al., 2003).

Other work contradicts the findings from these studies and suggests that RSV can cause intermittent acute infection in patients with COPD (Rohde et al., 2003). A study by Falsey and colleagues exploring the RSV persistence in COPD patients, detected RSV in just 0.95% of stable state sputum samples, however RSV was detected in 7.2% of exacerbation sputum samples (Falsey et al., 2006). This low prevalence of RSV in the stable state implies it is unlikely that RSV is persisting in these patients and acute infection is more likely to occur, perhaps leading to an exacerbation. Furthermore, Papi and colleagues found the prevalence of RSV to be 3.1% in stable COPD compared to 6.3% at exacerbation (Papi et al., 2006) and Rohde and colleagues found 22% of exacerbation samples to be positive for RSV but detected RSV in none of the stable state samples (Rohde et al., 2003) which again provides little evidence for the persistence of RSV.

The differences in RSV detection levels between these studies may be partly due to the methods used for viral detection, as well as differences in study samples. PCR is a sensitive technique that can detect low levels of viral prevalence and load. One consideration when addressing the variability in the field is whether PCR methods for RSV detection are too sensitive and that the RSV being detected actually plays no role in COPD pathogenesis. In 2001, Walsh and colleagues used a nested PCR method for the detection of RSV and found the nested PCR technique to be approximately 100-fold more sensitive than standard PCR and that the technique improved the ability of detecting RSV in respiratory samples (Walsh et al., 2001). It is important to consider, however, whether the RSV being detected by this method actually contributes to disease pathogenesis or whether it is harmless. Wilkinson and colleagues showed that patients positive for RSV in the stable state had worse lung function and higher airway inflammation than those without RSV which suggests that RSV does play a role in COPD pathogenesis and disease morbidity. Furthermore clustering of RSV detection in individuals as opposed to a random distribution of positive detections was also shown suggesting contamination was unlikely (Wilkinson et al., 2006a).

The results from the current study found no significant difference in the prevalence of RSV between the stable state, 21.9%, and exacerbation, 14.9% using unpaired analysis, however when separated into the individual RSV subtypes it was found that the prevalence of RSV-A was significantly higher in stable state samples compared to

exacerbation. This was not the case for RSV-B where the prevalence was significantly higher at exacerbation compared to the stable state however RSV-B was detected at a low prevalence in both disease states. By detecting RSV-A in almost a quarter of stable state samples, and with this prevalence being significantly higher than at exacerbation, this data supports the theory that RSV-A can persist within the airways of COPD patients and that low level chronic infection may persist in certain COPD patients. Patients are considered to be stable if exacerbation free for four weeks prior, and two weeks after the stable samples were collected, thus the RSV-A detected in the stable state samples was unlikely to be as a result of a previous exacerbation, or due to the start of a new one. In the paired analysis however, this was not the case, probably due to the relatively small sample size. There was no significant difference in the prevalence of RSV between the stable state and exacerbation, or in the prevalence of RSV-A between the two disease states, although in absolute numbers, the prevalence of RSV-A appeared to be higher in the stable state compared to exacerbation. RSV-B was not detected in any samples in the paired data set, and in only 0.5% of stable state and 2.5% of exacerbation state samples in the unpaired data set. These findings suggest that RSV-A is the most prominent subtype of RSV found in stable COPD and at exacerbation within the London COPD cohort, but given the variation in this field, these findings cannot be generalised to the population.

To date, there has been limited study into the relationship between RSV infection and the presence of upper respiratory tract (URT) symptoms such as cold symptoms and sore

throats, and lower respiratory tract (LRT) symptoms such as sputum volume and sputum purulence. Falsey and colleagues showed that patients positive for RSV reported symptoms such as cough, wheeze, dyspnoea, sore throats and cold symptoms with cough being the most common symptom reported (Falsey et al., 2006). In Chapter 5 an association between HRV infection and the presence of URT symptoms was described where, at exacerbation, HRV prevalence and load were found to be higher when associated with cold symptoms and/or sore throats, compared to when no symptoms were present, suggesting that URT symptoms could be used as potential markers of HRV infection. The current study showed no significant difference in the prevalence of cold symptoms, or sore throats in samples positive for RSV or samples negative for RSV from patients in the London COPD cohort, which suggests that URT symptoms would not be appropriate indicators of RSV infection in these COPD patients. Furthermore, unlike in the HRV data, there was no association between the prevalence of RSV and the number of symptoms present, whether it were cold symptoms alone, sore throats alone, or both symptoms together. Cold symptoms were significantly more prevalent in this data set compared to sore throats, or both symptoms, but this did not relate to the prevalence of RSV infection. When exploring LRT symptoms such as increases in sputum volume and increases in sputum purulence, there was also no difference between RSV positive samples and RSV negative samples in either of these symptoms. These findings suggest that symptoms should not be the preferred method of RSV infection indication and that, unlike HRV infection, the use of inflammatory markers may be more useful.

As RSV is considered, by some, to persist in COPD patients (Borg et al., 2003; Wilkinson et al., 2006a) rather than cause acute infection (Papi et al., 2006) leading to an exacerbation, it was important to explore whether, in the stable state, RSV infection was associated with levels of inflammatory markers as potential markers of RSV infection. In 2001, Seemungal and colleagues showed that when RSV was detected, mean fibrinogen levels and median serum IL-6 levels were elevated (Seemungal et al., 2001). In the current study, levels of CRP were explored to measure any differences between samples infected with RSV and those without RSV in order to determine whether CRP could be a potential candidate as a marker of RSV infection in stable COPD patients. The findings from the current study showed no difference in the levels of serum CRP between samples infected with RSV and samples not infected with RSV, suggesting that RSV presence in stable COPD does not cause an increase in CRP levels, implying that CRP would not be a useful marker of RSV infection. As previous studies have shown the levels of CRP to be significantly higher in the presence of bacteria compared to when bacteria was not detected (Garcha et al., 2012), it is likely that other inflammatory markers, more suitable for viral infection and that have not been explored in this study, may be more useful as indicators for RSV infection. In particular, as shown in this study, upper and lower respiratory tract symptoms are not suitable markers of RSV infection, and therefore, the availability of an inflammatory marker for this may be beneficial. Conversely, as RSV appears to play little role in the onset of COPD exacerbations, having an inflammatory marker to indicate RSV infection

may not be as important as for other viruses. Further study in this area would need to be performed for any conclusions to be made.

In the current study RSV load was measured using a semi-quantitative method which allowed comparisons to be made between individual sputum samples without the absolute load being known and is a way of performing relative quantification of loads rather than absolute quantification. When measuring viral load semi-quantitatively, a decrease in one Ct value represents a 2-fold increase in viral load. In the unpaired analysis, the detection threshold for RSV-A was found to be approximately 2-fold higher at exacerbation, relative to the stable state. The detection threshold for RSV-B was found to be approximately 16-fold higher at exacerbation, relative to the stable state. This suggests that the load of RSV-A and RSV-B was higher at exacerbation compared to the stable state however in order to make conclusions about changes in RSV load between these two disease states, the RSV load needs to be measured fully-quantitatively. In the paired analysis, there was no change in the detection threshold between the stable state and exacerbation.

Certain limitations of this study have prevented definite conclusions to be made; sample numbers, particularly in the paired analysis, are likely to have been too small resulting in the study being underpowered, particularly regarding exacerbation samples. Additionally, qPCR was used to semi-quantitatively detect RSV load. Although this method allowed

relative comparisons to be made between different samples in different disease states, it did not allow absolute viral loads to be compared and so it was therefore not possible to analyse or comment on differences in absolute RSV levels between samples. Further study into this field may focus on the absolute quantification of RSV load in stable COPD and at exacerbation allowing a better understanding of the role of RSV in COPD.

To date, antiviral therapy for RSV is of limited benefit but given the extent and severity of RSV infection in children, there is increasing interest in developing new treatments (Olszewska and Openshaw, 2009). Ribavirin and palivizumab are the only approved pharmacological agents for RSV treatment and prevention, respectively, and are predominantly used in children as there is limited data regarding their effectiveness in adults (Walsh, 2011). Specific antiviral therapy is often reserved for immunocompromised patients or those with severe respiratory failure. Therefore it seems that immunisation has the greatest potential for reducing RSV in adults, however, currently, there is no effective vaccine available for RSV (Walsh, 2011). In 1966, an RSV vaccine was tested in the United States, however the trial had serious consequences as many infants still caught RSV, some needing hospitalisation. Two infants died as a result of enhanced disease symptoms and since then, no safe vaccine has been developed (Delgado et al., 2009). The findings from this study show RSV to be present in both the stable state and at exacerbation, but at low prevalence, suggesting RSV is unlikely to play a significant role in causing exacerbations of COPD. Trials of anti-RSV therapy may be less beneficial in COPD

than in other situations, such as to treat infected infants, and also less beneficial than anti-viral trials for other viruses such as HRV, which has been shown in previous chapters to be closely associated with the onset and recovery of COPD exacerbations.

8.4 Conclusion

The findings from this study showed that the prevalence of RSV in sputum was not significantly different between stable COPD and exacerbation. In unpaired analysis, RSV-A prevalence was significantly higher in the stable state compared to exacerbation however this was not reproduced when using paired analysis. RSV-B prevalence was low in both disease states. The RSV-A load was found to be higher at exacerbation relative to the stable state but this was not the case for RSV-B. These findings suggest that RSV is unlikely to play a significant role in causing COPD exacerbations and that trials of specific RSV antiviral therapy may be less beneficial in COPD than antiviral therapies for other respiratory viruses which may play a more direct role in triggering COPD exacerbations.

Chapter 9. Summary and future research

9.1 Summary

The overall aim of this study was to investigate the relationship between human rhinovirus (HRV) infection and chronic obstructive pulmonary disease (COPD). It has been shown previously that HRV is the most commonly detected respiratory virus at COPD exacerbation (Seemungal et al., 2001) and with the development of qPCR techniques, it is possible to detect and quantify the levels of viral RNA in sputum samples from COPD patients. Few studies have investigated changes in HRV prevalence and load in COPD (Mallia et al., 2011; Quint et al., 2010; Seemungal et al., 2001), and there was an absence of literature exploring this virus at different points during COPD exacerbations using qPCR techniques. HRV infection over the entire time-course of COPD exacerbations and recovery had not been investigated in naturally occurring exacerbations.

A number of studies have explored the association of upper respiratory tract (URT) symptom changes and their recovery with viral-associated COPD exacerbations (Gerna et al., 2009; Seemungal et al., 2001; Wright et al., 2007). It has been shown that viral-associated exacerbations are more severe in terms of greater burden of symptoms and often have longer recovery times (Seemungal et al., 2001). However there has been no previous investigation into changes in HRV prevalence and load with URT symptoms. Furthermore, differences in the HRV load between patients with multiple URT symptoms compared to the load in those with just one symptom, has also not yet been explored.

Viral and bacterial co-infection in COPD is becoming important and more extensively studied (Mallia et al., 2012; Perotin et al., 2013; Wark et al., 2013). It has been shown that patients with both viral and bacterial infections have more severe COPD exacerbations compared to those with just one pathogen (Wilkinson et al., 2006) and are more likely to be readmitted to hospital following their exacerbation (Wark et al., 2013). Using experimental viral infection, it has been suggested that an initial viral infection can lead to the development of a secondary bacterial infection (Mallia et al., 2012) which can lead to further exacerbations. There have been no previous studies in naturally occurring exacerbations that have explored changes in both HRV load and bacterial load using qPCR over the entire time-course of an exacerbation and recovery. For the first time, this study reported changes in both prevalence and load of HRV and three typical and common airway bacteria *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* at exacerbation and during recovery.

Patient reported outcomes (PROs) are widely used in COPD to assess exacerbation severity and the effects of COPD on patient health status. To date, there has been no work into the association between PROs and HRV infection. Similarly, there has been extensive interest in the frequent exacerbator phenotype which has shown that exacerbations become more frequent and more severe as COPD severity increases and also that the more important determinant of exacerbation frequency is a history of exacerbations (Hurst et al., 2010; Seemungal et al., 1998). In 2001 Seemungal and colleagues showed a

relationship between HRV prevalence and exacerbation frequency (Seemungal et al., 2001b), however, to date, there has been no investigation into the relationship between HRV load and exacerbation frequency.

Respiratory syncytial virus (RSV) has been shown to be the second most commonly detected respiratory virus at COPD exacerbation, after HRV (Seemungal et al., 2001). There is much controversy about the role of RSV in COPD and whether it is able to persist long-term in the airways (Borg et al., 2003; Wilkinson et al., 2006) or whether it has a larger role in acute infection (Papi et al., 2006). In this thesis, the pilot study explored semi-quantitatively, the changes in RSV between stable COPD and exacerbation, and also changes in the individual RSV subtypes, RSV-A and RSV-B.

The findings of this study are summarised below.

9.2 Study findings

9.2.1 HRV prevalence and load in the stable state and at exacerbation

- The changes in HRV prevalence and load at COPD exacerbation and in the stable state were compared in *Chapter 3*. In a sputum sample population of 337 (58 stable state and 279 exacerbation), it was found that the prevalence of HRV was significantly higher at exacerbation compared to stable COPD. This was identified in both unpaired (41.9% vs 17%; $p=0.001$) and paired analysis (68.5% vs 16.7%; $p<0.001$).
- Similarly, it was shown in *Chapter 3* that HRV load was significantly higher at exacerbation compared to the stable state, in both unpaired ($p=0.008$) and paired analysis ($p=0.011$). In five patients in whom HRV was positive at both stable state and exacerbation, there was a significant increase in HRV load from $1.88(\pm 0.48)$ \log_{10} pfu/ml in the stable state to $4.01(\pm 0.68)$ \log_{10} pfu/ml at exacerbation; $p=0.034$.

9.2.2 Time-course of HRV infection during exacerbation and recovery

- *Chapter 4* explored the changes in HRV prevalence over the entire time-course of COPD exacerbation and recovery, using qPCR. HRV was detected at exacerbation presentation and Days 3, 7, 14, 35 and 56 post-exacerbation. In unpaired analysis, described in *section 4.3*, HRV prevalence was highest at exacerbation presentation and decreased significantly to each time point during the recovery period (all $p < 0.014$). Similarly HRV load was highest at presentation and decreased significantly to each time point following (all $p < 0.001$).
- It was shown in *section 4.4* using paired analysis, the prevalence was highest at presentation and decreased significantly to Day 14 ($p = 0.002$) and Day 35 ($p < 0.001$) post-exacerbation. The HRV load fell significantly from presentation to Day 3, 7, 14 and 35 (all $p \leq 0.043$) as shown in *Figure 4.4*.
- There was no significant difference between changes in inflammatory markers (IL-6, IL-8, IL-1 β , IL-18, TNF α , CRP, fibrinogen or IFN γ) over the time-course of an exacerbation and recovery between HRV-positive exacerbations and HRV-negative exacerbations as illustrated in *Figure 4.5*.

9.2.3 HRV infection and URT symptoms

- *Section 5.3.1* showed the prevalence of URT symptoms to be higher at exacerbation compared to the stable state (cold symptoms, $p=0.038$; sore throats, $p=0.026$). Furthermore the prevalence of URT symptoms was higher in the presence of HRV compared to without HRV (cold symptoms, $p<0.001$; sore throats, $p=0.002$).
- At exacerbation, HRV load was significantly higher in samples associated with URT symptoms compared to those without symptoms (colds, $p=0.004$; sore throats, $p<0.001$) as shown in *section 5.3.2*.
- In *section 5.5.2* it was shown that at Day 7 post-exacerbation, the prevalence of HRV was significantly higher when associated with cold symptoms compared to without symptoms ($p=0.016$). Similarly, at Day 3, the prevalence of HRV was significantly higher when associated with a sore throat compared to without ($p=0.036$).
- HRV load was significantly higher in samples associated with cold symptoms at exacerbation presentation, Day 3 and Day 7, compared to samples not associated with cold symptoms at these time-points ($p\leq 0.046$) as shown in *Figure 5.16*. HRV load was significantly higher in samples associated with sore throats at

exacerbation presentation and Day 3, compared to samples not associated with sore throats at these time-points ($p \leq 0.018$) as shown in *Figure 5.17*.

- In samples associated with both cold symptoms and sore throats, the HRV load was still significantly higher one week post-exacerbation compared to samples associated with just one of the symptoms (both $p < 0.030$) as shown in *Figure 5.18*.

9.2.4 Co-infection of HRV and typical airway bacteria

- *Chapter 6* showed that in exacerbations positive for HRV and bacteria at presentation, HRV load decreased during the time-course until zero at Day 14 (all $p < 0.001$). Bacterial load decreased from presentation to Day 7 ($p < 0.001$) but increased again at Day 14 ($p = 0.049$) as illustrated in *Figure 6.3*.
- In exacerbations positive for HRV but negative for bacteria at presentation, HRV load decreased during the time-course until zero at Day 14 ($p = 0.001$). Bacterial load increased significantly from presentation to Day 14 ($p < 0.001$) with 52% of bacteria negative samples becoming positive by Day 14 as illustrated in *Figure 6.8*.
- *H influenzae* was found to be the most prevalent bacterial species and was found at the highest loads throughout the time-course compared to *S pneumoniae* and *M catarrhalis* as shown in *section 6.3.2* and *section 6.5.2*.

9.2.5 HRV infection and patient reported outcomes

- In *section 7.3* it was shown that EXACT scores were significantly higher in the presence of HRV compared to without HRV ($48.4(\pm 1.33)$ vs $44.9(\pm 0.64)$; $p=0.010$).
- *Figure 7.4* showed that HRV load was significantly correlated with EXACT scores; EXACT scores increased as HRV load increased ($r=0.208$, $p=0.011$).
- EXACT scores were higher in the presence of cold symptoms ($p<0.001$) and sore throats ($p=0.021$) compared to without symptoms as described in *section 7.3*.
- *Section 7.5* showed that CAT scores were significantly higher in samples associated with HRV infection ($22.12(\pm 0.8)$) than those without ($19.95(\pm 0.5)$; $p=0.018$).
- CAT scores were weakly but significantly correlated to HRV load, increasing as HRV load increased ($r=0.149$, $p=0.003$) as shown in *Figure 7.10*.
- CAT scores were significantly higher in the presence of cold symptoms compared to without cold symptoms ($p<0.001$) but this did not reach statistical significant for sore throats as shown in *section 7.5*.
- It was shown in *section 7.7* that the median (IQR) number of exacerbations experienced per year was higher in patients with HRV infection, 3.01 (2.02-5.28) compared to those without HRV, 2.51 (1.88-3.51); $p=0.040$.

- As HRV load increased, exacerbation frequency increased showing a significant relationship between the two ($r=0.265$, $p=0.024$) as shown in *Figure 7.13*.

9.2.6 RSV infection in the stable state and at exacerbation

- *Chapter 8* showed no significant difference in the prevalence or semi-quantitative load of RSV between the stable state and exacerbation.
- When broken down into subtypes, the prevalence of RSV-A was shown to be significantly higher in the stable state compared to exacerbation as shown in *Figure 8.2*.
- *Section 8.3.2* showed no significant difference in the prevalence of URT symptoms between RSV-positive samples and RSV-negative samples at exacerbation, or in lower respiratory tract symptoms as shown in *section 8.3.3*.
- Levels of CRP were not significantly different between RSV-positive samples and RSV-negative samples in the stable state as shown in *Figure 8.11*.

9.2.7 Clinical implications of findings

- The findings from this study present strong evidence relating HRV infection to the onset of COPD exacerbations. Firstly, it has been shown that the prevalence of HRV increases significantly at exacerbation compared to in the stable state, as does HRV load, which provides strong evidence for the role of HRV in COPD exacerbations. Furthermore, by measuring the prevalence and load of HRV over the entire time-course of an exacerbation and recovery period, it was possible to follow the pattern of HRV infection and show that HRV was highest at exacerbation presentation and decreased significantly during recovery.

The finding that HRV load is highest at exacerbation presentation is an important development, as one of the obstacles in the consideration of antiviral therapy development is that it is considered that patients are likely to present to the clinic and be prescribed therapy too late for antivirals to be effective. However, this data shows that HRV is highest when patients present to the clinic suggesting treatment at the time of presentation may in fact be beneficial, which stresses the need for antiviral development for COPD exacerbations.

- At certain time-points during the time-course, HRV prevalence and load were higher in samples associated with URT symptoms compared to when no URT symptoms were reported. Furthermore, HRV prevalence and load were higher in

samples associated with both cold symptoms and sore throats compared to those associated with just one symptom only. The loads also remained higher for a longer period of time with the presence of more symptoms suggesting that exacerbations with more symptoms may be more severe and have longer recovery times than those with one symptom only. The fact that URT symptoms were reported a median of 2 days before exacerbation onset demonstrates how the London COPD cohort captures exacerbations early allowing early sampling and early treatment. These findings suggest that URT symptoms could be used as early indicators of HRV infection allowing treatment to be started earlier with the aim of reducing exacerbation severity or even preventing an exacerbation from occurring to start with. However, as some other respiratory viruses also cause similar symptoms, ultimately rapid diagnosis criteria, specifically for HRV, is required.

- This study showed that secondary bacterial infection occurs after initial HRV infection which may also have strong implications in terms of therapy at COPD exacerbation. Secondary bacterial infection may be one of the causes of recurrent exacerbations that can occur in some COPD patients. The results from this work further emphasise the importance of being able to treat HRV infections, not only to manage the HRV-associated exacerbation itself but also with the aim of preventing the development of a secondary bacterial infection which may then lead to another exacerbation. Whether antibiotic therapy would be required later into the

recovery period once a secondary bacterial infection had developed, would need further investigation, however, the accessibility of antiviral therapy for the treatment of HRV-associated exacerbations is crucial in reducing exacerbation frequency, severity and recurrence. One of the important features of secondary bacterial infection is that they may drive recurrent exacerbations and therefore follow up of COPD exacerbations is key, particularly at Day 14 after the onset of the event, in order to detect any recurrence. This is consistent with recommendations in the NICE quality standards for COPD, which suggests that all severe exacerbations need to be followed up 14 days after an exacerbation event (NICE Guidelines).

- An association between HRV infection and EXACT scores, and HRV and CAT scores was reported in this study. The results showed significantly higher EXACT and CAT scores in HRV infected subjects compared to those without HRV. Furthermore it was shown that as HRV load increases, EXACT and CAT scores also increase suggesting that these questionnaires could be used, alongside URT symptoms, to indicate HRV infection. This suggests the potential use of both questionnaires in early indication of HRV infection in COPD patients which may lead to appropriate treatment, early on at exacerbation onset. The EXACT questionnaire seems to be suitable for research studies, and studies of COPD exacerbations could be designed

with the EXACT as the primary outcomes, as it responds well to many aetiology factors of exacerbations.

- There was no significant difference in the prevalence of RSV between the stable state and exacerbation, however the prevalence of RSV-A was found to be significantly higher in the stable state compared to exacerbation. RSV-B was detected at a low prevalence in both disease states. These findings suggest that RSV is unlikely to play a significant role in causing COPD exacerbations so trials of anti-RSV therapy may be less beneficial in COPD than trials for other viruses that play a larger role in exacerbation onset, such as HRV.

9.3 Conclusion

This study has explored the role of HRV infection in chronic obstructive pulmonary disease, in the stable state, at exacerbation presentation and during exacerbation recovery, in naturally occurring COPD exacerbations. It has been shown that HRV prevalence and load are significantly higher at exacerbation compared to the stable state and decrease over the recovery period. HRV is associated with upper respiratory tract symptoms and patient reported outcomes and patients with HRV infection have higher exacerbation frequencies than those without HRV. Secondary bacterial infection is common after HRV infection which has important implications in terms of therapy and exacerbation recurrence. The treatment of respiratory viral infections in COPD patients, in particular HRV, may be fundamental in reducing exacerbation frequency. These findings emphasise the unmet need for the rapid development of therapeutic targets for both the prevention and treatment of HRV infection in patients with COPD.

9.4 Future work

- Although it has been shown that HRV prevalence and load increase at COPD exacerbation, it is not conclusive whether HRV is causing an exacerbation or is just contributing to the severity of an exacerbation once it has already arisen. Collecting sputum samples in the prodrome phase, prior to an exacerbation, will allow the role of HRV in these exacerbations to be further defined and thus determine to what degree it may be causative.

- In this study, HRV was identified using primers that are designed to detect as many serotypes of HRV as possible, to give a general view of the role of HRV in COPD. In terms of antiviral development, it is important to know what the most prevalent HRV serotypes involved in COPD exacerbations are, so sequencing HRV that is detected in sputum, would be useful for the development of strain-specific therapy. There are known difficulties in vaccine development for HRV due to there being over 100 serotypes, however knowing the main serotypes detected at COPD exacerbation may help in addressing this problem as vaccine development could then be aimed at targeting the most prevalent HRV serotypes.

- It has been shown that secondary bacterial infection is common after initial HRV infection; however in this study the mechanism by which this occurs has not been examined. Further study into the method of secondary bacterial infection development may provide further information in this area allowing any necessary changes to be made to therapeutic approaches.

- In 2001 Seemungal and colleagues reported HRV to be the most commonly detected virus at COPD exacerbation with RSV being the second (Seemungal et al., 2001a) which is why this study has focused on HRV infection and then addressed RSV to a lesser extent. Other viruses have also been detected at exacerbation, at lower prevalences such as influenza, parainfluenza and adenovirus, but may also play an important role in COPD exacerbations. These other viruses may also lead to the development of secondary bacterial infection in some patients. It is important to be aware of these viruses and the role they may play in COPD.

- As all patients in the secondary bacterial infection analysis were given antibiotics at exacerbation, it was not possible to determine any differences in the development of secondary bacterial infection between patients treated with antibiotics and those not treated. Further work may explore patients with HRV infection at exacerbation presentation, half of which are treated with antibiotics at

exacerbation and the other half given placebo. Of all patients in the study, 74% received oral prednisolone therapy and thus the effect of prednisolone on its own on secondary bacterial infection needs to be explored. This would allow any differences in secondary bacterial infection development between the two groups to be determined giving further insight into this area allowing appropriate changes to be made to treatment options if necessary.

- The results from this study have shown associations between HRV and various aspects involved in COPD. Without the development of therapy against HRV, HRV-associated exacerbations will continue causing increased morbidity and mortality in COPD patients, secondary bacterial infections will continue to occur resulting in recurrent exacerbations and increasing exacerbation frequency. These findings significantly emphasise the importance of rapid developments in therapeutic targets for the prevention and treatment of HRV infection in patients with COPD.

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Appendices

1. Original article - Human rhinovirus infection during naturally occurring COPD exacerbations (published in ERJ 2014).
2. Human rhinovirus load in stable COPD and at exacerbation (BTS 2011).
3. Changes in human rhinovirus load during naturally occurring COPD exacerbations: a prospective cohort study (ATS 2012).
4. The prevalence of clinically relevant micro-organisms in stable and exacerbated COPD using PCR techniques (ERS 2012).
5. Time-course of rhinovirus and bacterial infection during COPD exacerbation recovery (BTS 2012).
6. Human rhinovirus infection and secondary bacterial infection in COPD exacerbations (ATS 2013).
7. Human rhinovirus infection and exacerbation frequency at COPD exacerbation (BTS 2013).
8. Human rhinovirus infection and EXACT scores during COPD exacerbation (ATS 2014).
9. Time-course of human rhinovirus infection and upper respiratory tract symptoms during COPD exacerbations (BTS 2014).
10. Front of daily diary card
11. Back of daily diary card
12. EXACT questionnaire
13. CAT questionnaire